

Department of Production Animal Medicine
Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

**Canine dystocia –
oxytocin receptors, uterine inertia, and
cardiotocography**

Tuire Tamminen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Raisio Hall, Latokartanonkaari 7, Helsinki on December 18th 2020, at 12 noon.

Helsinki 2020

Director of studies	Professor Juhani Taponen, DVM, PhD Department of Production Animal Medicine Faculty of Veterinary Medicine University of Helsinki, Finland
Supervisors	Professor Emerita Terttu Katila, DVM, MS, PhD, Dipl. ECAR Department of Production Animal Medicine Faculty of Veterinary Medicine University of Helsinki, Finland Professor Juhani Taponen, DVM, PhD Department of Production Animal Medicine Faculty of Veterinary Medicine University of Helsinki, Finland Professor Outi Vapaavuori, DVM, PhD, Dipl. ECVS Department of Equine and Small Animal Medicine Faculty of Veterinary Medicine University of Helsinki, Finland
Reviewers	Professor Sandra Goericke-Pesch, DVM, PhD, Dipl. ECAR Reproduktionsmedizinische Einheit der Kliniken Stiftung Tierärztliche Hochschule Hannover, Germany Associate Professor Sabine Schäfer-Somi, DVM, PhD, Dipl. ECAR Besamungs- und Embryotransferstation Vetmeduni Wien, Austria
Opponent	Professor Alain Fontbonne, DVM, PhD, HDR, Dipl. ECAR Unité de Médecine de l'Élevage et du Sport (UMES) Alfort Ecole Nationale Vétérinaire, Paris, France

The Faculty of Veterinary Medicine uses the Urkund system (plagiarism recognition) to examine all doctoral dissertations.

ISBN 978-951-51-6810-8 (paperback)
ISBN 978-951-51-6811-5 (PDF)
<http://ethesis.helsinki.fi/>

Unigrafia Oy
Helsinki 2020

Cover photo: “Like mother, like son” by Chris Dutzi

*To my loved ones -
for all those moments lost.*

Abstract

This dissertation focuses on canine dystocia and is based on three individual studies conducted with client-owned pet animals. The aim was to elucidate the role of oxytocin receptors in uterine inertia. The suitability of human cardiotocography on parturition monitoring in bitches was tested.

Oxytocin is one of the most potent uterotonic hormones; its effect is mediated through specific oxytocin receptors, thus increasing the uterine contractions to expel the puppies through the birth canal. Oxytocin receptor distribution and expression in canine uterus were investigated with immunohistochemistry and qPCR. Progesterone prevents uterine contractions and prostaglandin F2 α has a key role in the initiation of parturition. Therefore, the role of progesterone, prostaglandin F2 α , as well as, oestradiol and ionized calcium in uterine inertia were investigated. The preparing contractions in the first stage of parturition are not externally visible, and the second stage of normal parturition may last up to 24 hours, which complicates the follow-up of the parturition in the bitch. Human cardiotocography was tested in uterine contraction and foetal heart rate monitoring to improve diagnosis of dystocia.

Oxytocin receptor expression and distribution were demonstrated in full-thickness uterine samples in non-pregnant, pregnant, and dystocic bitches; receptors were localized in all bitches in the endometrial luminal, superficial and deep glandular epithelium, in the stroma, and in the longitudinal and circular myometrium. The relative expression of oxytocin receptors was higher in pregnant than in non-pregnant bitches. Progesterone concentrations were significantly higher in samples taken prior to the first stage of parturition in group of elective Caesarean section bitches than in obstructive dystocia or combined dystocia groups. Prostaglandin F2 α metabolite concentrations were significantly higher in bitches with normal parturition than in bitches of elective Caesarean section or combined dystocia groups. Ionised calcium concentrations did not differ between the groups and no hypocalcaemia was detected.

The absence of progesterone as such cannot be the only stimulation for oxytocin receptor expression in uterus, because the anoestrous basal level progesterone did not upregulate the oxytocin receptors. The gradual decrease of progesterone in late pregnancy is more likely involved in oxytocin receptor upregulation than sudden prepartum drop, because the upregulation of oxytocin receptors seems to occur before the sudden prepartum decline of progesterone. The decline of oestrogens two days prior to parturition may have regulatory role in oxytocin receptor expression. Our results suggest that in complete primary uterine inertia the aetiology is not the absence or downregulation of oxytocin receptors but more likely in the uterine functional level.

The cardiotocography complements clinical and ultrasonographic examination of periparturient bitches. It detects uterine contractions and can be used to confirm diagnosis of uterine inertia and to monitor the response to uterotonic medicine treatment. The cardiotocography's ultrasonographic doppler probe appeared to be sensitive to artefacts, which may compromise foetal distress monitoring.

Acknowledgements

The studies were carried out at the University of Helsinki, Faculty of Veterinary Medicine, Department of Production Animal Medicine, Department of Equine and Small Animal Medicine, and at the Karolinska Institutet in Stockholm. Financial support was provided by the Finnish Foundation of Veterinary Research, the Finnish Veterinary Foundation, and the European Veterinary Society for Small Animal Reproduction (EVSSAR).

I owe my deepest gratitude to the director and supervisor of the studies, Juhani Taponen, and to supervisors Terttu Katila and Outi Vapaavuori. Their constructive critique, advice, and patience over the years have carried me through this journey. The initial idea of the study was by Outi Vapaavuori who was an opponent of Annika Bergström on her thesis of Canine Dystocia. I also thank Magnus Andersson and Merja Dahlbom for the fruitful discussions during the process of writing the manuscripts.

The staff of the Veterinary Teaching Hospital of the University of Helsinki kindly assisted in sample collection, even in the most inconvenient hours in the middle of the night, thank you. The blood samples were run in the Central Laboratory of Veterinary Teaching Hospital with the kind assistance of Merja Pöytäkangas and Satu Sankari, thank you for sharing your knowledge and time and for your help. Antti Flyckt, Jere Linden and Reijo Jokivuori were invaluable in taking and editing the photomicrographs, thank you. Uterine samples were prepared in Department of Veterinary Biosciences; thank you, Kati Holmsten, for organising everything. The IHC and qPCR were run in Karolinska Institutet in Stockholm. Thank you, Lena Sahlin and Britt Masironi-Malm, who with open arms took me to learn the methods in their laboratory and patiently explained the principles and practice of the methods used.

I am sincerely grateful to Sandra Goericke-Pesch and Sabine Schäfer-Somi not only for reviewing this dissertations's manuscript but also for their constructive criticism, valuable corrections, and suggestions that improved the paper. Carolyn Abbot edited the English language, thank you.

This study would not have been possible without the cooperation of the dog breeders, especially Jonna Laitinen, Kirsti Määttänen, and Anu Purola, who allowed their bitches to participate in the study and provided all required details about their bitches. You are golden, thank you!

I will enormously miss discussions with colleagues during the lunch and coffee breaks in Saari Unit. It has been a privilege to have you all around giving inspiration, thank you. With the support of my friends, sharing the high spirit days and the darkest moments, I have reached the goal. A big hug to you, you know who you are!

Finally, I owe my greatest gratitude to my family for being there, standing beside me – without you, I would not be me.

Aakkulanharju, Tampere 19.7.2020



Contents

Abstract	5
Acknowledgements	7
List of original publications.....	10
Abbreviations	11
1 Introduction	12
2 Review of the literature.....	14
2.1 <i>Normal parturition</i>	<i>14</i>
2.1.1 Initiation of parturition.....	14
2.1.2 Stages of parturition.....	17
2.1.3 Key hormones of parturition in bitch	18
2.1.3.1 Progesterone	18
2.1.3.2 Prostaglandin F2 α	18
2.1.3.3 Oxytocin.....	18
2.2 <i>Oxytocin receptor</i>	<i>19</i>
2.3 <i>Uterine contractility</i>	<i>21</i>
2.3.1 Uterus and uterine contractions	21
2.3.2 Methods to study uterine contractions.....	22
2.3.2.1 Cardiotocography	22
2.4 <i>Dystocia.....</i>	<i>23</i>
2.4.1 Occurrence of dystocia.....	23
2.4.2 Causes of dystocia	24
2.4.2.1 Uterine inertia	24
3 Aims	26
4 Materials and methods.....	27
4.1 <i>Ethical permission</i>	<i>27</i>
4.2 <i>Research sites.....</i>	<i>27</i>
4.3 <i>Animals and study groups</i>	<i>27</i>
4.4 <i>Blood samples</i>	<i>29</i>
4.4.1 Progesterone.....	30
4.4.2 PGF2 α metabolite.....	30
4.4.3 Oestradiol	31
4.4.4 Ionised calcium.....	31
4.5 <i>Uterine samples.....</i>	<i>31</i>
4.5.1 Histology.....	32
4.5.2 Oxytocin receptors, PCR.....	32
4.5.2.1 RNA preparation and reverse transcription	32
4.5.2.2 qPCR analysis	32
4.5.3 Oxytocin receptors, IHC.....	33

4.6 Cardiotocography.....	34
4.7 Statistical analysis.....	35
5 Results	36
5.1 Hormones and ionised calcium (II)	36
5.2 Uterine samples	38
5.2.1 Histology (I)	38
5.2.2 Oxytocin receptors, qPCR (I, II).....	38
5.2.3 Oxytocin receptors, IHC (I, II)	42
5.3 Cardiotocography (III)	45
6 Discussion	50
6.1 The uterus of the non-pregnant bitch	50
6.2 Regulation of oxytocin receptors in bitches	51
6.3 Uterine inertia	52
6.4 Cardiotocography in canine parturition monitoring	54
6.5 Limitations of the study.....	56
6.6 Further studies	57
7 Conclusions	60
References	61
Original articles	71
Appendices	

List of original publications

This thesis is based on the following original publications:

- I Tamminen, T.M., Sahlin, L., Masironi, B., Taponen, J., Laitinen-Vapaavuori, O., Katila, T. (2017). Oxytocin receptors in dioestrous and anoestrous canine uteri. *Reprod. Domest. Anim.* 52:153-159.
- II Tamminen, T., Sahlin, L., Masironi-Malm, B., Dahlbom, M., Katila T., Taponen, J., Laitinen-Vapaavuori, O. (2019). Expression of uterine oxytocin receptors and blood progesterone, 13,14-dihydro-15-Keto-Prostaglandin F2 α , and ionized calcium levels in dystocic bitches. *Theriogenology*. 135, 38-45.
- III Tamminen, T., Dahlbom, M., Katila, T., Laitinen-Vapaavuori, O., Taponen, J. Monitoring of uterine contractions in the bitch. Submitted.

The publications are referred to in the text by their Roman numerals. In addition, some unpublished data are also presented. Original publications are reproduced with the kind permission of the copyright holders.

Abbreviations

ADH	antidiuretic hormone (vasopressin)
CL	corpora lutea
COMB1	combined groups: CUI, PUI, OD, MD
COMB2	combined groups: CUI, PUI, OD
CS	Caesarean section
CTG	cardiotocography
CUI	complete primary uterine inertia
ECS	elective Caesarean section
EDTA	ethylenediaminetetraacetic acid
EHG	electrohysterography
E2	oestradiol
GE	endometrial glandular epithelium
iCa	ionized calcium
IHC	immunohistochemistry
LE	endometrial luminal epithelium
LH	luteinizing hormone
MD	medically treated dystocia
MYO	myometrium
mRNA	messenger ribonucleic acid
NORM	normal parturition
OHE	ovariohysterectomy
OD	obstructive dystocia
OXT	oxytocin
OXTR	oxytocin receptor
PCR	polymerase chain reaction
PRE	prepartum
PUI	partial primary uterine inertia
PGE2	prostaglandin E2
PGFM	13,14-dihydro-15-Keto-Prostaglandin F2 α , prostaglandin F2 α metabolite
PGF2 α	prostaglandin F2 α
P4	progesterone
qPCR	quantitative (real-time) polymerase chain reaction
RIA	radioimmunoassay
RNA	ribonucleic acid
TBS	tris buffered saline

1 Introduction

Dystocia is a common complication of parturition in bitches; it increases the risk of puppy mortality and may threaten the life of the dam (Darvelid and Linde-Forsberg 1994; Münnich and Küchenmeister 2009). Diagnosis of canine dystocia may be challenging, as the preparing contractions in the first stage of parturition may not be visible, and the second stage of normal parturition may last up to 24 hours (Johnston et al. 2001; Linde-Forsberg 2010). Approximately 75% of the reasons for maternal dystocia are because of uterine inertia (Darvelid and Linde-Forsberg 1994). Any reason that prolongs the parturition may disturb the contractility of the uterus, which makes uterine inertia a complex problem.

Oxytocin (OXT), a nonapeptide hormone, is released from the posterior pituitary gland into systemic circulation following a suitable stimulus, such as cervical pressure during parturition (Haterius and Ferguson, 1938; Ferguson 1941). The effect of OXT in the target tissue is mediated by specific G-protein coupled transmembrane receptors, oxytocin receptors (OXTR). In the uterus, the binding of OXT to its receptors leads to uterine contractions, if the myometrium is capable of contracting.

OXT and OXTR have a crucial role in uterine contractility, so many studies of OXT and OXTR are conducted widely in several species (cow, human, mouse, pig, rat, sheep) and different organs and tissues (Zingg et al. 1995; Gimpl and Fahrenholz 2001; Zingg and Laporte 2003; Arrowsmith and Wray 2014). Gene expression of OXT and OXTR is regulated in many species, such as cow (Fuchs et al. 1992), pig (Lundin-Schiller et al. 1996), and sheep (Beard and Lamming 1994; Wathes et al. 1996), by an increase of oestrogens and a decrease of progesterone. Uterine distention increases OXTR expression, even in non-pregnant uterine horn in rats (Ou et al. 1998).

Studies of OXTR in canine uterus are sparse, but it has been shown that the expression of OXTR is higher in the uterus of pregnant bitches near term compared with earlier stages of pregnancy, which indicates that OXTR are upregulated at the time of prepartum luteolysis (Derussi et al. 2012; Gram et al. 2014, Veiga et al. 2015). Antigestagen treatment in mid-pregnancy increased expression of OXTR in the uterus of pregnant bitches (Gram et al. 2014). These studies indicate that progesterone (P4) has a regulatory role in OXTR expression in dogs, but basic research is needed to clarify the regulation of OXT and OXTR systems in non-pregnant and pregnant bitches to understand the mechanisms behind uterine contractility.

Cardiotocography has been used in humans for decades to follow up labour (Ayres-de-Campos et al. 2015). It has also been used in dogs, but few reports exist (Davidson 2001; Schröder et al. 2006; Groppetti et al. 2010). The method is based on external detection of abdominal pressure changes and provides more precision to evaluate the frequency, amplitude and duration of the contractions.

The purpose of the study was to investigate dystocia, especially the mechanisms behind uterine inertia and uterine contractility in bitches. Relevant hormones (progesterone, prostaglandin F2 α metabolite, oestradiol), ionized calcium in the blood, and the distribution and expression of OXTR in the uterus of anoestrous, dioestrous, prepartum and peripartum bitches were analysed to gain further insights into their role in parturition and dystocia. The suitability of cardiotocography was tested to improve diagnostics of dystocia in the bitch.

2 Review of the literature

2.1 Normal parturition

2.1.1 Initiation of parturition

Parturition is a complex event and includes hormonal and behavioural changes, neural activity, and interaction between the dam and the offspring. The initiation of parturition is a cascade of events that are presented in common farm animals in Figure 1. Dogs are similar in these events, but several recent studies indicate that the regulatory mechanisms of luteal function are unique in this species. However, there are still open questions. Figure 2. describes the main hormonal regulatory mechanisms of luteal function in dogs. Kowalewski et al. (2015) describes the importance of locally produced prostaglandins to the development of the CL in the bitch: Formation of the CL is associated with increased prostaglandin E₂ (PGE₂) and low prostaglandin F₂ α (PGF₂ α) luteal content. PGE₂ also seems to have an indirect luteotrophic role by stimulating the expression of prolactin receptor (Kowalewski et al. 2015). The canine CL does not require gonadotrophic support at first, during the relative gonadotrophin independence time, but several potential factors, such as immune system components together with cytokines, are suggested to maintain CL (Hoffmann et al. 2004; Mariani et al. 2006). Prolactin is the main gonadotrophic factor to maintain CL during the gonadotrophin dependent period, from 25 days after preovulatory LH peak onwards (Okkens et al. 1986). The events leading to prepartum luteolysis differ greatly from those initiating luteolysis in non-pregnant dogs. In the pregnant dog, shortly before parturition, PGF₂ α is released from the utero-placental unit leading to a sudden decrease in serum P₄ concentration 24 h before parturition (Luz et al. 2006; Kowalewski et al. 2010, Gram et al. 2013, Gram et al. 2014b).

A functional foetal hypothalamo-pituitary-adrenal axis is obligatory to the initiation of parturition. Over 70% damage in a sheep's foetal pituitary gland led to a ewe's failure to initiate parturition (Liggins et al. 1967). Preparturient environmental stress induces foetal adrenocorticotrophic hormone (ACTH) secretion and a cortisol level increase, leading to the removal of the progesterone (P₄) block that prevents uterine contractions (Taverne and Noakes 2018). The increase of prepartum foetal cortisol secretion has been demonstrated in sheep (Alexander et al. 1968, Liggins et al. 1973) and dogs (Jackson and Piasecki 1969, Jackson et al. 1973) species. Higher cortisol levels were recently found in allantoic than in amniotic fluids in periparturient bitches (Bolis et al. 2017), which likely indicates increased foetal cortisol secretion. In bitches, maternal peripheral plasma cortisol levels increase 8 to 24 hours before parturition (Concannon et al. 1978; Hoffmann et al. 1994; Veronesi et al. 2002; Olsson et al. 2003). However, cortisol levels vary greatly between individuals, and the increase is more associated with parturition itself and stress,

not as a part of the initiation cascade (Hoffmann et al 1994). Increasing plasma PGF2 α metabolite (PGFM) levels in prepartum bitches indicate increased release of PGF2 α that leads to luteolysis, followed by a decrease in peripheral P4 levels that allow contractions of the uterus and progression of parturition (Concannon et al. 1988; Nohr et al. 1993; Hoffmann et al. 1994; Veronesi et al. 2002). Expression of the P4 receptor mRNA is highest in the canine uterus during the pre-implantation period and lower during the rest of the gestation; it is suggested that decreased expression of the P4 receptor activates utero-placental prostaglandin synthesis and prepartum PGF2 α release (Kowalewski et al. 2010, 2014b).

Plasma levels of the antidiuretic hormone (ADH) (Olsson et al. 2003) and oxytocin (OXT) (Olsson et al. 2003; Klarenbeek et al. 2007) increase peripartum in the bitch. An increase of ADH may also be associated with the stress and pain of labour in bitches, because it is recognised in ewes (Kendrick et al. 1991) and heifers (Hydbring et al. 1999). Higher pressure to the cervix in rabbits increases OXT release (Haterius and Ferguson 1938; Ferguson 1941). Oestradiol (E2) concentration is somewhat higher in the last trimester of pregnancy in the bitch, but there is no marked prepartum increase as detected in many other species, such as cow (Robertson 1974), rat (Yoshinaga et al. 1969), and sheep (Robertson and Smeaton 1973). Two days prior to parturition, the oestrogen levels of the bitch decrease suddenly during prepartum luteolysis, indicating its luteal source (Concannon et al. 1975; Hoffmann et al. 1994).

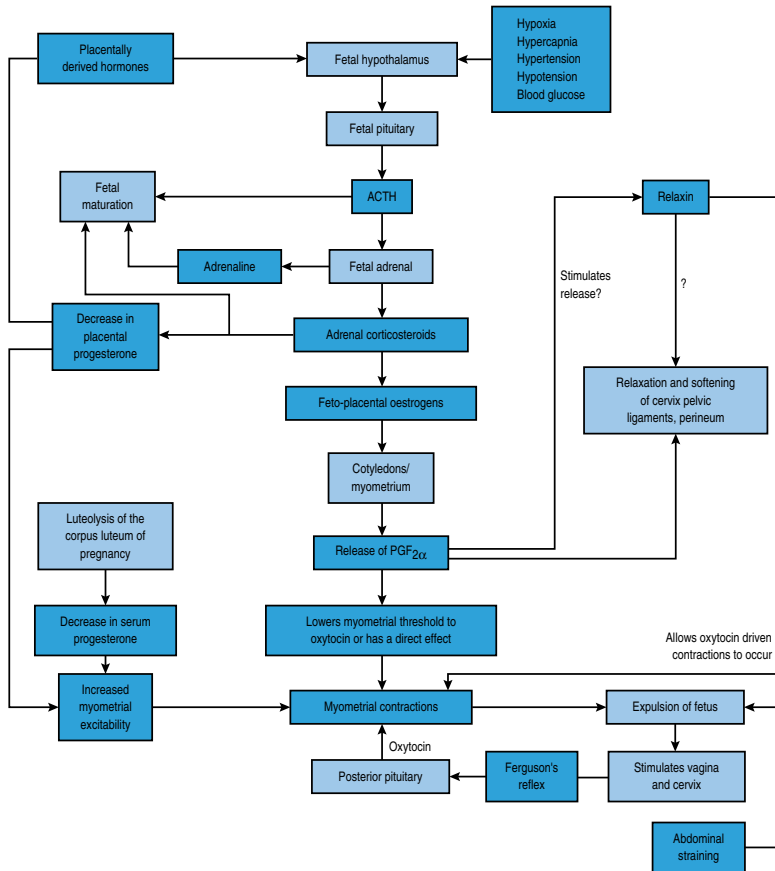


Figure 1. Pre- and periparturient endocrinological events in cows, ewes and sows. Obtained from Taverne and Noakes (2019).

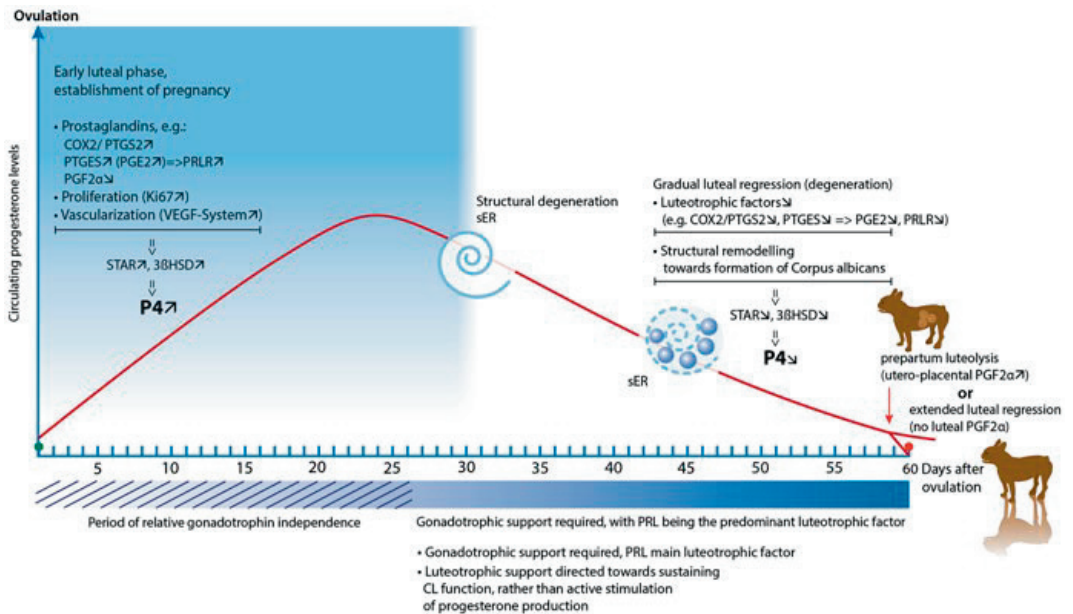


Figure 2. Main hormonal regulation mechanisms of luteal function in bitches. COX2/PTGS2, cyclooxygenase 2 (PTGS2); PTGES, PGE2 synthase; PRLR, prolactin receptor; STAR, steroidogenic acute regulatory protein; 3 β HSD (HSD3B2), 3 β -hydroxysteroid- dehydrogenase; sER smooth endoplasmic reticulum; VEGF vascular endothelial growth factor. Obtained from Kowalewski et al. (2015).

2.1.2 Stages of parturition

Canine pregnancy lasts 64–66 days from luteinising hormone (LH) surge or 62–64 days from ovulation (Concannon et al. 1983). Gestational length may be longer, at least in some breeds, when the number of puppies is 4 or less (Eilts et al. 2005). At the time of luteolysis, the body temperature typically drops at least 1 °C from the baseline (Concannon et al. 1989). The parturition progresses gradually from one stage to another. The description of stages presented here is based on the works of Johnston et al. (2001) and Linde-Forsberg (2010). The first stage of parturition is the preparing time for the birth canal and foetuses. The cervix starts to dilate, and myometrial contractions are initiated. The contractions are weak and intermittent and not externally visible. Typical changes in behaviour are decreased appetite, restlessness, panting and obsessive nest building. The first stage lasts approximately 6–12 hours but can, especially in nervous, primiparous bitches, be prolonged to 36 hours. The cervix is fully dilated in the second stage, and uterine contractions become

stronger, forcing the puppies to pass through the birth canal. The puppy stimulates the dam to assist the birth by straining her abdominal muscles when it is entering the birth canal. The body temperature has risen back to the baseline. The second stage lasts approximately 6 hours, but it can extend to 24 hours. The rest of the foetal membranes and placentas are expelled in the third stage. Most membranes and placentas are delivered with the puppy or from 5 to 15 minutes after birth. Sometimes placentas can be retained.

2.1.3 Key hormones of parturition in bitch

2.1.3.1 Progesterone

The maintenance of pregnancy in bitches depends on P4 secretion from corpora lutea (CL) throughout the gestation, and ovariectomy anytime during pregnancy induces abortion (Sokolowski 1971, Tsutsui 1983). Plasma P4 declines abruptly (close to or below 6 nmol/L) approximately 24 hours before parturition (Concannon et al. 1988; Nohr et al. 1993; Hoffmann et al. 1994; Veronesi et al. 2002). Non-pregnant bitches have similar P4 levels in dioestrus as pregnant ones, but the decline of P4 is not as sudden as seen before parturition (Concannon et al. 1975). It seems that at the end of dioestrus, the CL regress by a degenerative process without any luteolytic agent (Kowalewski et al. 2014, 2015). Prepartum luteolysis is a more active process in which PGF2 α increases and local, intraluteal processes are involved (Kowalewski et al. 2014, 2015).

2.1.3.2 Prostaglandin F2 α

The major metabolite of PGF2 α is 13,14-dihydro-15-keto-prostaglandin F2 α , which is easier to measure because it has a longer half-life in peripheral circulation than PGF2 α (Ginther et al. 2007). Plasma PGFM starts to increase 24–48 hours before parturition; it decreases again to the preparturient level after delivery (Concannon et al. 1988; Veronesi et al. 2002; Olsson 2003). The prepartum increase in secretion of PGF2 α in the bitch is suggested to originate from placental trophoblast cells (Luz et al. 2006; Kowalewski et al. 2010, Gram et al. 2013, Gram et al. 2014b).

2.1.3.3 Oxytocin

Oxytocin (OXT) is a neuropeptide hormone of nine amino acids (Fig. 3) produced in hypothalamic paraventricular and supraoptic nuclei and stored in the posterior pituitary gland (Gimpl and

Fahrenholz 2001; Arrowsmith and Wray 2014). It is released into systemic circulation after an appropriate stimulus, such as intracervical pressure known as Ferguson's reflex (Haterius and Ferguson, 1938; Ferguson 1941). OXT is also synthesised to some extent in peripheral tissues: for example, heart (rat); prostate gland (guinea pig, rat, dog, human); ovaries (pig, monkey, cow) (Gimpl and Fahrenholz 2001). OXT, one of the most potent uterotonic hormones, enhances the contractility of the uterus (Gimpl and Fahrenholz 2001; Arrowsmith and Wray 2014). Plasma OXT concentration increases during canine parturition, (Olsson et al. 2003; Klarenbeek et al. 2007), however there is a large variation between individuals.

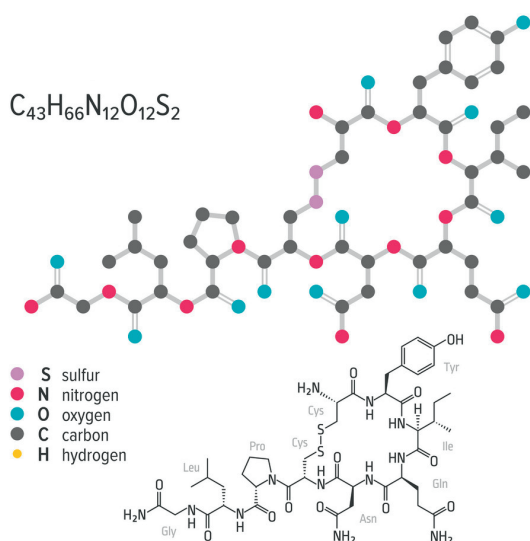


Figure 3. The oxytocin molecule structure. Obtained from VectorStock.

2.2 Oxytocin receptor

Oxytocin receptor (OXTR) belongs to a group of rhodopsin-type (Class 1) G protein-coupled receptors that are common receptors in mammals (Zingg et al. 1995; Gimpl and Fahrenholz 2001; Zingg and Laporte 2003; Arrowsmith and Wray 2014). OXTR has seven transmembrane domains; the agonist binding region of the receptor is different from the antagonist binding site (Gimpl and Fahrenholz 2001; Zingg and Laporte 2003). Figure 4 presents the structure of OXTR.

Several organs, in addition to the uterus and the mammary gland, express OXTR (Gimpl and Fahrenholz 2001; Zingg and Laporte 2003; Arrowsmith and Wray 2014), which indicates that OXT has a versatile role in the entire body. Uterine OXTR has been studied widely in the rat (Fuchs and Soloff 1983; Ou et al 1998), cow (Fuchs et al. 1992; Robinson et al. 1999, 2001), sheep (Wu et al. 1996), horse (Starbuck et al. 1998), and pig (Kitazawa et al. 2001) species.

Uterine OXTRs are upregulated before the onset of labour, and uterine sensitivity to OXT increases (Gimpl and Fahrenholz 2001; Arrowsmith and Wray 2014). The function and regulation of the OXTR system is strongly steroid dependent; the increase of OXTR mainly depends on a decrease in P4 and an increase in E2 in several species, such as cow (Fuchs et al. 1992), pig (Lundin-Schiller et al. 1996), and sheep (Beard and Lamming 1994; Wathes et al. 1996). Uterine OXTR expression in sheep is similarly increased in ewes with cortisol-induced and spontaneous parturition (Wu and Nathanielsz 1994). Continuous exposure to OXT in humans leads to desensitisation of OXTR by reducing the OXT binding sites in the myometrial cell membrane and by downregulating the OXTR messenger ribonucleic acid (mRNA) in myometrial cells (Phaneuf et al. 1997). Uterine distention seems to have a role in OXTR expression. Stretching a non-pregnant horn in rats induces a high periparturient expression of OXTR, similar to pregnant horn (Ou et al. 1998).

Recent studies of OXTR in the canine uterus have shown that the expression of OXTR mRNA is higher in the uterus of pregnant bitches near term compared with earlier stages of pregnancy, indicating that OXTRs are upregulated at the time of parturition (Derussi et al. 2012; Gram et al. 2014; Veiga et al. 2015). Antigestagen treatment in mid-pregnancy increased the expression of OXTR mRNA in the uterus of pregnant bitches (Gram et al. 2014). These studies indicate that progesterone has a regulatory role in OXTR expression in dogs, but further investigation is required to clarify the regulatory mechanisms.

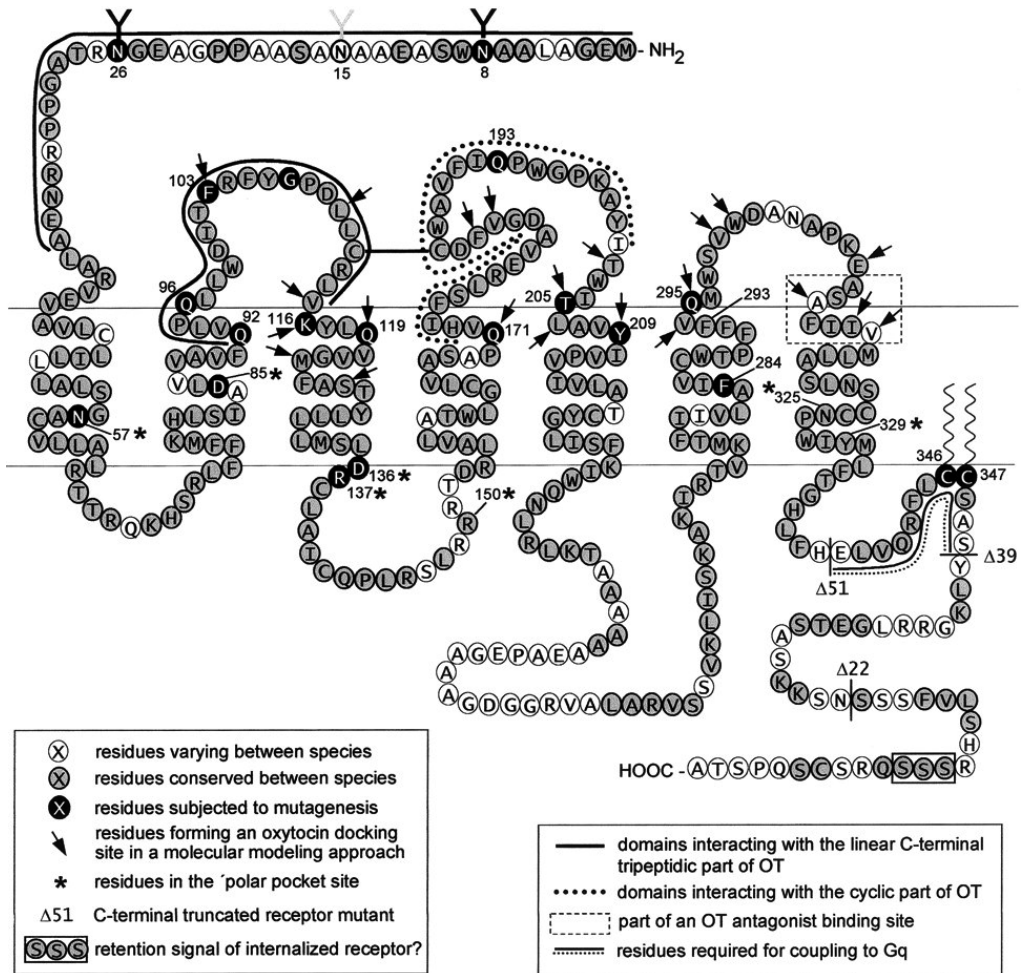


Figure 4. The structure of the oxytocin receptor. Obtained from Gimbl and Fahrenholz (2001).

2.3 Uterine contractility

2.3.1 Uterus and uterine contractions

The canine uterus is bicornuate with long horns and a common cervix (Nickel et al. 1979). Uterine contractions are produced by the myometrium, which is divided into inner circular and outer longitudinal layers (Nickel et al. 1979). The main contractile components of myometrial smooth muscle cells are thin actin and thick myosin filaments (Aguilar and Mitchell 2010). These protein

filaments form covalent crosslinked bonds that lead to the contraction of myofibrils, which is the myometrial contraction (Aguilar and Mitchell 2010). OXT-induced myometrial contractions in rats depend on the influx of extracellular calcium, and this influx is directly increased by OXT (Batra 1986). OXT inhibits the ionized calcium (iCa) extrusion pump in humans (Popescu et al. 1985). Sarcoplasmic reticulum regulates concentrations of iCa (Aguilar and Mitchell 2010; Vannuccini et al. 2016). OXT increases intracellular iCa levels, phosphorylation of myosin, myosin-actin binding, and secretion of PGF2 α ; these events enhance myometrial contractions (Vannuccini et al. 2016).

2.3.2 Methods to study uterine contractions

Canine uterine contractions during oestrus and mating have been demonstrated with uterine fistula (Tsutsui et al. 1989), uterine-implanted, catheter-tip pressure transducers (Wheaton et al. 1988; Ibuki et al. 1997), and M-mode ultrasound (England et al. 2006). Uterine contractions prepartum and peripartum have been recorded with electromyography (van der Weyden et al. 1989). Ultrasound, a non-invasive method, could be useful also in contraction monitoring during parturition, but there are no reports of such use in dogs. The other methods are not applicable to parturition monitoring in clinical work.

2.3.2.1 Cardiotocography

Cardiotocography (CTG) has been used for decades to monitor uterine contractions and foetal distress in women. The follow up may be intermittent or continuous depending on the stage and the labour's risks (Ayres-de-Campos et al. 2015). Interpretation of CTG in human medicine, includes observation of the foetal heart frequency: basal heart rate level, changes in the rate in the short and long term, and acceleration and deceleration of the rate (Ayres-de-Campos et al. 2015). The frequency, intensity, and duration of the uterine contractions, as well as changes in foetal heart rate in relation to uterine contractions are followed (Ayres-de-Campos et al. 2015). External monitoring of uterine contractions with CTG is not directly quantitative but gives information of the relative contraction strength, duration, and frequency. The frequency of contractions correlates well with methods of monitoring intrauterine pressure, but the strength and duration of contractions may be inaccurate, especially in obese human patients (Miles et al. 2001; Ayres-de-Campos et al. 2015). The intrauterine pressure catheter is considered the most reliable method in human medicine (Gibb, & Arulkumaran, 1987). However, this method can be used only after the rupture of foetal membranes, and it is more invasive than CTG.

Tocography of CTG measures uterine contractions with a specific extrauterine probe. The method is based on measuring actual or relative changes in pressure through the abdominal muscles, skin, and hair. A doppler probe that records the foetal heartbeats is connected to CTG. Pressure changes and the foetal heart rate are shown electronically in a screen and printed as a graph. The tocographic probe is positioned cranially to the navel or slightly laterally to the midline of the abdomen and kept in place with an elastic belt with the aim to focus on the fundus of the uterus (Euliano et al. 2013).

In the United States, Whelpwise™ Veterinary Perinatal Specialities® offers services and equipment for veterinarians and breeders to monitor parturition at home. Uterine contractions are recorded with an extrauterine method, tocodynamometry, also known as tocography. A doppler probe is also included to monitor the foetal heart rate. However, few reports exist about cardiotocography in dogs (Davidson 2001; Schröder et al. 2006; Groppetti et al. 2010).

2.4 Dystocia

2.4.1 Occurrence of dystocia

Dystocia means difficult birth or inability to expel puppies through the birth canal. It is a common complication of canine parturition and increases the risk of puppy mortality (Darvelid and Linde-Forsberg 1994; Münnich and Küchenmeister 2009; Cornelius et al. 2019). Uterine contractions reduce the placental blood flow, thus decreasing oxygen supply to the foetus (Assali et al. 1958; Sato et al. 2016), which may predispose to hypoxia and compromise the survival of the newborn in prolonged parturitions. Success in delivering live offspring depends on the structure and the size of the dam's pelvic cavity, sufficient ripening of the cervix, adequate ability of the uterus to contract to expel the puppies through the birth canal, and the duration of the parturition. Survival of the litter also depends on the size, number, conformation, and vitality of the puppies. A cohort study of 224 breeds in Norway found that puppy mortality was influenced by breed, increasing age of the bitch, and litter number and size, although there was great variation between individual litters (Tønnessen et al. 2012). Occurrence of dystocia in bitches varies greatly depending on the population studied; the average is estimated to be below 5% (Linde-Forsberg, 2010). Dystocia occurred in 16% of parturitions in a group of 200, 000 insured bitches (excluding Boston Terrier, English Bulldog, and French Bulldog) in Sweden (Bergström et al. 2006). The occurrence varied from 0% to 92% among 151 breeds in the UK (22, 005 litters) (Evans and Adams 2010). Other data from the UK state a dystocia prevalence of 3.7% in an entire emergency-case population of 18,758 bitches (O'Neill et al. 2017). Dystocia seems to be more common in miniature and small breeds (Gaudet 1985; Bergström et al. 2006; Münnich and Küchenmeister 2009), but several medium- and large-size

breeds also have a higher than average proportion of litters by Caesarean section (CS) (Evans and Adams 2010). Two guide dog colonies (2,489 litters) of small (< 5 puppies) and large (> 9 puppies) litter sizes resulted in a higher dystocia rate than in a medium size litter (Cornelius et al. 2019). The same study found that the increasing age of the bitch increased the dystocia risk. A study of 701 dystocia cases showed that the odds of dystocia were increased in bitches weighing under 10.0 kg (1.6 times) and increased even more in bitches weighing 40.0–49.9 kg (3.5 times), when compared with bitches weighing 20.0–29.9 kg (O'Neill et al. 2017). Approximately 60% of dystocia cases undergo CS (Gaudet 1985; Darvelid and Linde-Forsberg 1994; Bergström et al. 2006; Münnich and Küchenmeister 2009). The proportion of CS is very high, 80–90% of parturitions, in Boston Terrier, English Bulldog and French Bulldog breeds (Evans and Adams 2010). A bias risk may exist in the statistics of dystocia in these breeds due to the popularity of elective CS (ECS).

2.4.2 Causes of dystocia

The definition of suggested causes of dystocia varies slightly depending on the authors (Johnston et al. 2001; Linde-Forsberg 2010; Davidson 2010). Sometimes there are several simultaneous causes of dystocia. Maternal factors are more common than foetal ones. According to the study of Darvelid and Linde-Forsberg (1994), the most common maternal cause is primary uterine inertia, which can be complete (48.9%) or partial (23.1%). Less common causes are a narrow birth canal (1.1%), uterine torsion (1.1%), and uterine hernia. According to Darvelid and Linde-Forsberg (1994), the most common foetal causes are malpresentation (15.4%), foetal oversize (6.6%) and malformation (1.6%). Environmental disturbance may delay parturition, but it can resume if the disturbance ends and is not too detrimental to the bitch (Bleicher 1962). Plasma P4:PGFM concentration ratio was lower in dystocic bitches than in control ones in Bergström et al.'s study (2010).

2.4.2.1 Uterine inertia

Uterine inertia has been classified according to the clinical situation as primary or secondary and complete or partial (Johnston et al. 2001; Linde-Forsberg 2010; Davidson 2010). However, the aetiology of uterine inertia is complex, and any reason that prolongs the parturition may disturb the contractility of the uterus. The uterus fails to initiate uterine contractions in complete primary uterine inertia (CUI); hence, no puppies are born (Darvelid and Linde-Forsberg 1994). In a typical case, the gestation period is over and foetal fluids have passed, but the parturition is not progressing in cases of primary uterine inertia as in normal parturition to the second stage (Johnston et al. 2001;

Linde-Forsberg 2010). OXT concentrations may be lower in cases of primary uterine than in normal parturition (Bergström et al. 2006, Bergström et al. 2010). There may be an inherited predisposition to uterine inertia (Linde-Forsberg 2010), because breeders can recognise lines where inertia occurs more commonly than others. Failure in luteolysis is suspected to lead to prolonged gestation (Irons et al. 1997), which may lead to premature detachment of the placenta and foetal death. In small litters with one or two puppies, the stimulation for the initiation of parturition may be inadequate (Linde-Forsberg 2010). The myometrium is unable to contract due to weakness or overstretching in very large litters (Johnston et al. 2001; Linde-Forsberg 2010). In partial primary uterine inertia, the bitch may have weak uterine contractions or contractions that cease without any obvious reason, such as obstruction, before all the puppies are born (Darvelid and Linde-Forsberg 1994). A hormonal or nutritional imbalance may lead to uterine inertia (Linde-Forsberg 2010). Medical treatment of uterine inertia may be attempted with calcium gluconate or calcium gluconate (10% solution with 0.465 mEq Ca^{2+} /ml) and OXT (Johnston et al. 2001; Davidson 2010; Linde-Forsberg 2010); calcium increases the strength and OXT the frequency of contractions (Davidson 2010).

3 Aims

The study's overall aim was to elucidate the mechanisms behind dystocia, especially in uterine inertia, the contractility of the uterus, and the role of oxytocin receptors. Specific aims were as follows:

- Investigate the role of progesterone, prostaglandin F2 α metabolite, oestradiol, ionised calcium, and oxytocin receptors in bitches with and without dystocia.
- Localise oxytocin receptors in the canine uterus with immunohistochemistry in non-pregnant anoestrous and dioestrous bitches and in pregnant bitches near term and during dystocia.
- Investigate the relative expression of oxytocin receptors in the canine uterus with and without the influence of progesterone.
- Investigate the relative expression of oxytocin receptors in the uterus of pregnant bitches near term (elective Caesarean section) and during Caesarean section in bitches with dystocia.
- Test the suitability of human cardiotocography for monitoring canine parturition, especially in diagnosing uterine inertia.

4 Materials and methods

4.1 Ethical permission

Blood sampling from bitches with normal parturition in Study II was authorised by the National Animal Experiment Board (ESAVI, Hämeenlinna, Finland), license number ESAVI/3802/04.10.03/2011. The other parts of the studies were approved by the Ethics Committee of the Viikki Campus, University of Helsinki, Finland. The bitches' owners were requested to give their oral consent in Studies I and III and to sign a written consent in Study II to use the material and data obtained from the bitches.

4.2 Research sites

The studies were conducted at the University of Helsinki, Faculty of Veterinary Medicine, Department of Production Animal Medicine and Department of Equine and Small Animal Medicine. The samples were collected in the Veterinary Teaching Hospital and in a private small animal clinic nearby. Blood iCa was analysed at the Veterinary Teaching Hospital. Hormonal assays were run in the Central Laboratory of the Department of Equine and Small Animal Medicine. Uterine samples were prepared in the Department of Veterinary Biosciences. The immunohistochemistry (IHC) and quantitative PCR (qPCR) were run in the Karolinska Institutet in Stockholm, Sweden.

4.3 Animals and study groups

All bitches participating in the studies were client-owned pet animals representing several breeds.

In Study I, the inclusion criteria were ovariohysterectomy (OHE) without signs of pyometra and an oral consent from the owner to use the removed tissue. The bitches ($n = 17$) were divided in two groups, anoestrus ($n = 9$) and dioestrus ($n = 8$), according to the stage of the oestrous cycle (Table 2). Pro-oestrous and oestrous bitches were excluded as well as bitches with ovarian abnormalities.

In Study II, the inclusion criterion was a diagnosis of dystocia resulting in CS or elective CS (ECS) due to a small litter size or previous dystocia, or medically treated dystocia. A control group consisted of bitches with normal parturition. The following study groups were formed from altogether 58 bitches:

1. Complete primary uterine inertia (CUI; n = 7)
 - no puppies born, parturition does not proceed, discharge of foetal fluids at least 3 hours or green discharge, no response to vaginal stimulus
2. Partial primary uterine inertia (PUI; n = 13)
 - at least one puppy born, parturition ceases without obstruction
3. Obstructive dystocia (OD; n = 10)
 - foetal oversize or narrow birth canal, malpresentation, malformation
4. Elective Caesarean section (ECS; n = 11)
 - 58-66 days from mating, previous dystocia or one or two puppies, before the onset of the stage 1 of parturition
5. Medically treated dystocia (MD; n = 8)
 - no CS, medical treatment
6. Control (C; n = 9)
 - no CS, no medical treatment, normal parturition

Dystocia groups were also combined (COMB1: CUI, PUI, OD, MD and COMB2: CUI, PUI, OD) to compare with ECS and C. The diagnosis and treatment decisions were made by the veterinarian on call. After blood sampling, the bitches were treated, if necessary, with calcium glubionate (Calcium-Sandoz®, Sandoz A/S, Copenhagen, Denmark) and oxytocin (Vetox®, Vetcare, Salo, Finland). The bitches' owners were requested to complete a questionnaire to obtain their bitch's history, including previous and present parturitions (Appendices). The onset of the first parturition stage was assessed on the available information about the following changes: drop of body temperature by at least 1 °C; decreased appetite; restlessness; panting; and obsessive nest building. The beginning of the second parturition stage, indicated by the dam's abdominal straining and rupture of foetal membranes, was considered the end of the first stage. Any systemic disease was an exclusion criterion.

In Study III, altogether 47 client-owned pregnant bitches were monitored at home or in a private veterinary clinic before the onset of parturition and during parturition (Table 4). The bitches' body weight was from 5 to 65 kg, the mean being 20.6 kg (± 15.1). Their age was from 2 to 6 years, the mean being 4.1 years (± 1.2). The mean number of puppies was 6.1 (± 2.7). The monitors were also tested in 5 non-pregnant individuals to detect the effect of body movements on pressure changes. Their weight was from 7 to 21 kg, the mean being 12.8 kg (± 5.9), and the age was from 4 to 8 years, the mean being 6.2 years (± 1.2). Number of monitoring sessions of each

bitch was from 1 to 5. The aimed monitoring time was 30 minutes at a time, but it depended on the bitch's cooperation. Prepartum monitoring was timed from 55 to 61 days after ovulation and from 58 to 62 days from mating. Monitorings represented the prepartum time, the first and the second stage of normal parturition, and the different types of dystocia, as follows: prepartum (PRE, n = 13); during the first (FIRST, n = 7) and the second (NORM, n = 17) stages of normal parturition; in CUI (n = 3); in PUI (n = 8); in OD (n = 3); and in MD (n = 6). The criteria in the dystocia groups were the same as in Study II. The bitches were treated, if necessary, with oxytocin (Vetox®, Vetcare, Salo, Finland, 1–4 IU im). Monitorings were performed after oxytocin administration in 12 bitches (six in PUI, six in MD).

4.3.1 Stage of the oestrous cycle

Study I determined the stage of the oestrous cycle by the history of the last oestrus, inspection of vulvar oedema and discharge, macroscopical findings of ovaries, vaginal cytology, and serum P4 concentrations as defined by Christie et al. (1972) and Concannon et al. (1975). A vaginal smear consisting mainly of parabasal and intermediate epithelial cells, P4 concentration below 6 nmol/L, no vulvar oedema or discharge, and no follicles present in ovaries indicated anoestrus. A vaginal smear containing < 30% of cornified superficial cells, P4 concentration over 6 nmol/L, no vulvar oedema or discharge, and no follicles but CL present in ovaries were indicative of dioestrus. The vaginal smear and P4 analysis were not performed in one case. That bitch had been mismated once during a visibly normal heat 19 days before OHE. Embryos or placentation sites were detected neither in the examination of uterine lavage fluid with light microscopy nor in the visual inspection of the uterus after incision. Several CL were seen in the ovaries. The bitch was included in the dioestrous group. A bitch with ovarian tumour was excluded from the study.

4.4 Blood samples

Blood samples were taken from the cephalic vein (Table 1) into a syringe (Radiometer Safe Pico, ref: 956-610, Radiometer Medical, Copenhagen, Denmark), into an ethylenediaminetetraacetic acid (EDTA) tube (Vacuette®, Mekalasi Oy, Nurmijärvi, Finland,) with 5000 KIU aprotinin/ml EDTA blood (Aprotinin, Roche Diagnostics GmbH, Mannheim, Germany), and into a serum tube with clotting activator (Vacuette®, Mekalasi Oy, Nurmijärvi, Finland). EDTA tubes and syringes were stored in an ice-water bath and serum tubes at room temperature. EDTA and serum samples were centrifuged (Eppendorf Centrifuge 5810R, Eppendorf Nordic A/S, Hørsholm, Denmark) as

follows: EDTA tubes at 4 °C, 1200 x g, 10 min, and serum tubes at 22 °C, 1700 x g, 10 min. Plasma and serum were divided into aliquots, frozen at -20 °C, and stored at -70 °C until analysed.

Table 1. Blood sample type and timing in Studies I and II.

	Reproductive status of the bitch	Sampling time	Sample type	Analysis
Study I	Anoestrus or dioestrus	Prior to OHE	Serum	P4
Study II	Prepartum (ECS) or peripartum (CUI, PUI, OD, MD, C)	Prior to CS or medical treatment (MD) or during 2nd stage of parturition (C)	Serum	P4, E2
			EDTA plasma with enzyme inhibitor	PGFM
			Safe Pico syringe	iCa

OHE, ovariectomy; P4, progesterone; ECS, elective Caesarean section; CS, Caesarean section; E2, oestradiol; CUI, complete primary uterine inertia; PUI, partial primary uterine inertia; OD, obstructive dystocia; MD, medically treated dystocia; C, control; PGFM, prostaglandin F_{2α}; iCa, ionised calcium.

4.4.1 Progesterone

Serum P4 concentrations were measured in one run (Studies I and II) using a commercial radioimmunoassay (RIA) kit (Progesterone Coat-A-Count® RIA, Siemens Healthcare Diagnostics Oy, Espoo, Finland) according to the manufacturer's instructions. The concentrations were measured in duplicate with a gamma counter (1272 GliniGamma, LKB Wallac Oy, Turku, Finland). The intra-assay coefficient of variation was 3.4% at a serum concentration of 4.4 nmol/L and 2.0% at a concentration of 32.5 nmol/L. The detection limit was 0.3 nmol/L.

4.4.2 PGF_{2α} metabolite

Study II measured concentrations of PGFM, from plasma using a commercial immunoassay kit (DetectX® 13,14-dihydro-15-keto-PGF_{2α} Enzyme Immunoassay Kit, Arbor Assays, Michigan, USA) according to the manufacturer's instructions. Prior to performing the assay, plasma samples were diluted 1:15 with the assay buffer provided in the kit. The optical density of each well was measured with a Multiscan GO Spectrophotometer with SkanIt software 4.1 (Thermo Fisher Scientific Oy, Vantaa, Finland). The intra-assay coefficient of variation of duplicates was 12.0%. The inter-assay coefficient of variation was 13.0% at a plasma concentration of 15.8 nmol/L and

3.5% at a concentration of 58.5 nmol/L. The linearity of the assay was evaluated by diluting the canine plasma sample (1/10, 1/20 and 1/40) with the assay buffer provided in the kit. Observed to expected ratios were calculated for the dilutions. The mean recovery of the expected PGFM concentrations in different dilutions was 102% and dilutions of the canine plasma sample showed linearity over the studied range ($R^2 = 0.996$). The detection limit was 0.13 nmol/L.

4.4.3 Oestradiol

In Study II, concentrations of 1,3,5(10)-Estratriene-3,17 β -diol; 17 β -oestradiol were measured from serum using a commercial RIA kit for human serum or plasma (Ultra-sensitive estradiol RIA, Beckman Coulter DSL4800, Immunotech, Prague, Czech Republic) according to the manufacturer's instructions. The concentrations were measured in duplicate with a gamma counter (1272 GliniGamma, LKB Wallac Oy, Turku, Finland). The theoretical sensitivity according to the manufacturer was 8.14 pmol/L.

4.4.4 Ionised calcium

In Study II, blood iCa was analysed instantly with Roche Electrolyte Analyzer (9180, Fisher Scientific Oy, Vantaa, Finland) from Safe Pico syringes stored in an ice-water bath. The syringes contained 60 IU of dry electrolyte-balanced heparin. Contact with air was minimised with a specific cap to remove possible air bubbles.

4.5 Uterine samples

In Study I, immediately after the removal of the ovaries and uterus, a full thickness sample was taken from the uterine body and from the middle part of the horn. In Study II, uterine samples were obtained from bitches undergoing a CS. Immediately after the removal of the puppies from the uterus, a full-thickness sample of uterine wall was taken from the incision site (interplacental area, uterine body or proximal horn). The sample (approximately 5 x 30 mm) was divided into two parts. One part was immediately frozen in liquid nitrogen and stored at -70 °C for PCR analysis (studies I and II). The other part was fixed in 4% phosphate-buffered formaldehyde (pH 7.0) for a minimum

of 20 hours and maximum of 24 hours and subsequently stored in 70% ethanol at +4 °C until processed further with an automated tissue processor (Tissue-Tek VIP™ 5 Jr. Vacuum Infiltration Processor, Sakura, Algol Diagnostics, Espoo, Finland). Sections (5-µm) were mounted on slides (SuperFrost Plus, Thermo Scientific, Gerhard Menzel GmbH, Braunschweig, Germany) for IHC (Studies I and II) and histological evaluation (Study I).

4.5.1 Histology

In Study I, uterine sections on slides were stained with haematoxylin-eosin for histological evaluation. Endometrial cysts were recorded. The number of leucocytes was estimated in vessels in myometrium and endometrium as being negative (-), occasional in a few fields (+), occasional in several fields (++) and several in all fields (+++).

4.5.2 Oxytocin receptors, PCR

4.5.2.1 RNA preparation and reverse transcription

The samples of Studies I and II were handled and run simultaneously. The uterine samples were relocated from -70 °C to -20 °C and after 24 hours transferred to RNeasy®Lysis Buffer (Qiagen Inc., Austin, Texas) and stored at -20 °C until cut into 25–30-mg pieces and homogenised. Total RNA from the uterine homogenate was isolated and purified using the RNeasy® Mini kit (Qiagen GmbH, Hilden, Germany) according to the procedure for RNA isolation from fibrous tissues, including a DNase step, as recommended by the manufacturer. Concentration and purity of RNA were determined spectrophotometrically at 260 nm and 280 nm. The RNA quality was assessed by visualisation of 28S and 18S rRNA bands after electrophoresis. A 2-µg aliquot of total RNA from each uterine sample was reverse transcribed at 37 °C for 60 min in a final volume of 20 µl with a reaction mixture (Qiagen, Hilden, Germany) containing 1 × RT buffer, dNTP mix (0.5 mM each dNTP), 600 ng random primers (Invitrogen, Paisley, UK), 2 units of RNase inhibitor (Qiagen, Hilden, Germany), and 4 units of Omniscript™ reverse transcriptase (Qiagen, Hilden, Germany).

4.5.2.2 qPCR analysis

The oligonucleotide primer pair for the OXTR was designed with NCBI/Primer-BLAST. To standardise the quantification method, RPL27 and HPRT1 were selected as non-regulated reference genes and the primer pairs were obtained from Silva et al. 2009 and Bhatti et al. 2007, respectively. The primers were based on the sequences of the canine genes. The primers used were OXTR forward primer: 5'-TGCTGGCCTTCATCGTGTGCT-3'; OXTR reverse primer: 5'-GATGAAAGCCGAGGCTTCCTTGGG-3' from NM_001198659.1 with a predicted size of 95bp; RPL27 forward primer: 5'-ACAATCACCTCATGCCCACA-3'; RPL27 reverse primer: 5'-CTTGACCTTGGCCTCTCGTC-3' from NM_001003102.2 with a predicted size of 122 bp; HPRT1 forward primer: 5'-AGCTTGCTGGTGAAAAGGAC-3'; HPRT1 reverse primer: 5'-TTATAGTCAAGGGCATATCC-3' from NM_001003357.1 with a predicted size of 104 bp.

Real time PCR was performed in an iCycler™ iQ Real Time PCR System (Bio-Rad Laboratories Inc., Stockholm, Sweden). For PCR, the cDNAs corresponding to 50 ng (HPRT1) or 100 ng (OXTR and RPL27) RNA were added to 12.5 µL of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Stockholm, Sweden), and 0.3 µM of each oligonucleotide primer was added to a final volume of 25 µL. After an initial incubation for 3 min at 95 °C, the samples were subjected to 40 cycles of 10 s at 95 °C, followed by 45 s at 57 °C. All samples were run in duplicate. The purity of PCR products was confirmed by a melting curve analysis in all experiments. Each PCR assay included a negative control containing an RNA sample without reverse transcription. The PCR amplification rate and the cycle threshold values were analysed using iCycler™ iQ 3.1 software (Bio-Rad Laboratories Inc., Stockholm, Sweden). The OXTR product was normalised against the mean of RPL27 and HPRT1 products to give the relative expression of OXTR mRNA.

4.5.3 Oxytocin receptors, IHC

The samples of Studies I and II were handled and run simultaneously for IHC. Uterine sections on slides were deparaffinised in xylene, rehydrated in a graded ethanol series and rinsed with distilled water and tris buffered saline (TBS). The sections were boiled in sodium citrate (0.01M, pH 6.0) in a microwave oven for 10 minutes. After cooling 20 minutes at room temperature and rinsing with TBS, the sections were covered with 3% H₂O₂ in methanol to block endogenous peroxidase. After rinsing with TBS, a commercial blocking reagent (Background Sniper, Biocare Medical LLC, Concord, CA 949520, USA) was used to prevent non-specific binding. As primary antibody, a purified polyclonal rabbit-anti-human antibody to OXTR (ABIN302438, Antibodies Online, Aachen, Germany) was used in 1:200 dilution with TBS. For negative controls, the primary antibody was replaced by an equivalent amount of rabbit IgG. After incubation for one hour at room temperature and subsequent rinsing with TBS and 0.01% Tween20, a biotinylated horse-anti-rabbit

antibody was added to sections as a secondary antibody in 1:500 dilution with 5% normal horse serum in TBS containing 5% BSA and incubated in the dark for 30 minutes. After rinsing with TBS and 0.01% Tween20, avidin biotin complex (Vectastain® Elite ABC Kit, PK6100, Vector Laboratories Ltd, Peterborough, United Kingdom) was added and sections were incubated in the dark for 30 minutes to enhance signals. After rinsing with TBS and 0.01% Tween20, DAB chromogen substrate (DAKO K3466) was added to achieve brown staining. After rinsing with distilled water, haematoxylin was added for counterstaining. Sections were rinsed with running tap water, dehydrated in a graded ethanol series and xylene.

Immunostaining of OXTR was assessed by manual scoring in myometrial (longitudinal and circular layers) smooth muscle cells (MYO) and in luminal epithelial cells (LE) and endometrial superficial and deep glandular epithelial cells (GE) of the endometrium. The blind scoring was performed independently by two observers on a four-point scale from negative (0), faint (+), moderate (++) to strong (+++) immunostaining.

4.6 Cardiotocography

In Study III, two human CTG monitoring units (Sonicaid FM 820 and TeamCare, Algol Diagnostics, Vantaa, Finland) were used to detect uterine contractions and foetal heartbeats. In tocography, also known as tocodynamometry, uterine contractions are measured with a specific extrauterine probe. The method is based on measuring relative changes in pressure through abdominal muscles, skin, and hair. A doppler probe that records the foetal heartbeats is connected to the monitoring unit. Pressure changes and the foetal heart rate are shown electronically on a screen and printed as a graph.

The tocographic probe was placed caudal to the costal arch and lateral to the midline of the abdomen and fixed in place with a flexible belt. At the onset of the monitoring, the level of the pressure was zeroed to set the basal level (10% units). The reset to zero was repeated when necessary: if at the beginning the adjustment to zero took place during a uterine contraction or if the position or tightness of the belt was corrected later. Changes in the bitch's posture were followed (probe under the bitch, the bitch sitting, standing, or moving). Also, general nervousness or relaxation were recorded (the bitch being relaxed, panting, or having nervous movements). Paper printing speed for tocographs was 1 or 2 cm/min. When printing was not possible because of a technical problem or running out of paper (13 monitoring sessions out of 60), the amplitude and frequency of contractions were recorded manually from the screen. The relative pressure change (amplitude changes above 10% units), and number, duration (> 1 minute), and interval of the contractions were counted. Occasionally, uterine activity below limits set (amplitude change below

10% units, contraction duration < 1 minute) was noticed, but these were not counted. Foetal heartbeats were measured with the doppler probe whenever possible. If necessary, the position of the probe was determined by localising the heartbeats with a stethoscope. Hair was clipped when necessary and ultrasound gel was used. Both monitors were used in all groups, and tocographic scale and patterns were similar.

4.7 Statistical analysis

The data were analysed using IBM SPSS Statistics 23 (Study I) and 24 (Study II, III) software for Windows. Differences were considered statistically significant at $p < 0.05$.

Study I: The non-parametric Related-Samples Wilcoxon Signed Rank Test was used for the staining intensity in IHC and relative expression of OXTR mRNA between the horn and the body of the uterus and between dioestrous and anoestrous uteri. The correlation of serum P4 and expression of OXTR mRNA was evaluated with Kendall's Tau.

Study II: The non-parametric Kruskal-Wallis one-way ANOVA test with Bonferroni correction was used to detect possible differences in serum P4 levels, plasma PGFM levels, blood iCa, and relative expression of OXTR mRNA between the groups.

Study III: The non-parametric Kruskal-Wallis one-way ANOVA test with Bonferroni correction was used to detect differences between the groups in the relative pressure change (amplitude change in contraction graph, %-units), in the number of contractions per 10 minutes, and in the duration of the contractions.

5 Results

5.1 Hormones and ionised calcium (II)

Blood samples were taken prior to the 1st stage of parturition in ECS group, during the 2nd stage and prior to possible medical treatment and CS in dystocia groups (CUI, PUI, OD), during the 2nd stage and prior to medical treatment in MD group, and during the 2nd stage in C group. P4, PGFM, E2, and iCa were measured in all individuals in Study II.

Serum P4 concentrations were numerically highest prior to the 1st stage of parturition in the ECS group; the largest variation in levels was also noticed in this group. The concentrations were significantly ($p < 0.05$) higher in the ECS than in the OD or in the COMB1 groups (Fig. 5).

Plasma PGFM concentrations were numerically highest in the 2nd stage of parturition in the C group and lowest prior to the 1st stage of parturition in the ECS group. A significant difference ($p < 0.05$) was detected between the C and the ECS and between the C and the COMB1 groups. Numerically P:PGFM ratio was highest in the ECS group. A significant difference ($p < 0.05$) was observed between the ECS and the C and between the ECS and the COMB1 groups (Fig. 5).

In blood samples taken from bitches just before the 1st stage or during the 2nd stage of parturition, most of the E2 levels were under the detection limit. The standard curves were acceptable, and a control sample from an oestrous bitch resulted in expected levels. These results were not used for further evaluation.

Blood iCa concentrations did not differ between bitches prior to the 1st stage (ECS group) and during the 2nd stage of parturition (dystocia and C groups) (Fig. 5). No hypocalcaemia (reference interval 1.16–1.40 nmol/L) was detected.

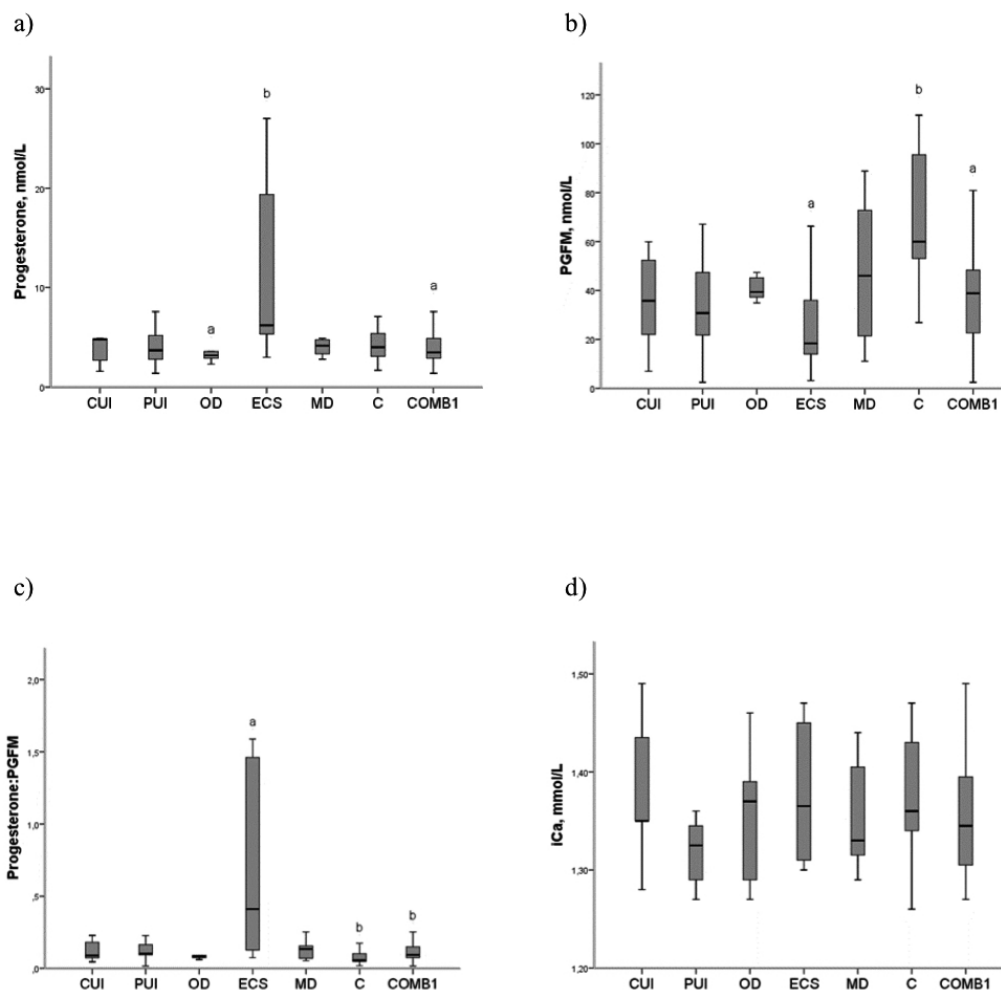


Figure 5. Concentrations of hormones and ionized Ca (iCa) in blood samples of prepartum, periparturient, and dystocic bitches; a) serum progesterone, b) plasma prostaglandin F2 α metabolite (PGFM), c) progesterone:PGFM ratio, d) blood iCa. The boxplots show the median with 50% of the data falling within the box. The whiskers extend to the 5th and 95th percentiles. Boxes with different letter designations are significantly different at $p < 0.05$. CUI, complete uterine inertia; PUI, partial uterine inertia; OD, obstructive dystocia; ECS, elective Caesarean section; MD, medically treated dystocia; C, normal parturition; COMB1, four dystocia groups combined: CUI, PUI, OD, MD. (Study II)

5.2 Uterine samples

Full thickness uterine samples were collected from anoestrous and dioestrous bitches after OHE for Study I, and from pregnant bitches during CS for Study II. In Study I, uterine samples were taken from body and from the middle part of the horn, and from the incision site (interplacental area, uterine body or proximal horn) in Study II. The summary of the individual and average data of Study I is presented in Table 2 and Study II in Table 3.

5.2.1 Histology (I)

All layers of uterine tissue were present in all 17 horn and in 16 body samples. In one case, the sample of the body was not available. In six samples, vacuolated cytoplasm, due to lipid accumulation, was noticed in the luminal epithelium and in the crypts of the endometrium (4 dioestrous, 2 anoestrous). Uterine hyperaemia, cysts and fibrotic changes were found in a few cases (Table 2). Fewer crypts and smaller glands were noticed in anoestrous than in dioestrous endometrial samples.

5.2.2 Oxytocin receptors, qPCR (I, II)

In Study I, no significant difference was detected in relative expression of OXTR mRNA between the horn and the body, or between anoestrous and dioestrous uteri. There was no correlation between OXTR mRNA expression and the blood progesterone concentration. In study II, the mean relative expression of OXTR mRNA was highest in the ECS group (Fig. 6). The difference was significant ($p < 0.05$) between the ECS and the OD and between the ECS and the COMB2 groups. There was no significant difference between bitches treated or not treated with calcium glubionate and OXT. The relative expression of OXTR mRNA was significantly ($p < 0.05$) higher in the uterus of pregnant bitches, in both COMB2 and ECS, than in non-pregnant ones (Fig. 6)

Table 2. The individual and average data and results for the 17 bitches divided into anoestrus and dioestrus groups in Study I. Macroscopical ovarian (CL: corpora lutea, CLr: regressed corpora lutea) and histological uterine (endometrial cysts, leucocytes in endometrium and myometrium) findings are presented. The immunohistochemical (IHC) staining for oxytocin receptors (OXTR) is scored in the uterine horn (H) and body (B) in the myometrium (MYO), and in the endometrial luminal (LE) and glandular epithelium (GE). P4, progesterone; n/a, not available.

Breed Age (years) Weight (kg)	P ₄ nmol/L	CL or CLr	Stage of oestrous cycle	Comments	Leucocytes: endo/myo	Endo- metrial cysts	IHC scoring for OTR in uterine horn (H) and body (B)					
							H MYO	H LE	H GE	B MYO	B LE	B GE
Airedale Terrier 1.7, 24.6	0.4	-	anoestrus	Uterine hyperaemia	+/+		0	0	2	n/a	n/a	n/a
Alaskan Husky 6.2, 23.7	0.4	CLr	anoestrus	Uterine hyperaemia	+++	+	2	3	3	0	1	2
Chihuahua 5.4, 2.9	1.4	CLr	anoestrus	Uterine hyperaemia	+++	+	1	3	3	0	1	1
Hovawart 7.3, 33.4	1.0	CLr	anoestrus	Mammary tumours	+/+		2	2	3	2	2	3
Mixed 1.0, 7.7	1.1	-	anoestrus		+/+		3	1	3	0	1	2
Mixed 2.1, 24.6	0.9	CLr	anoestrus		+/+		3	1	3	2	1	3
Mixed 2.4, 36.4	0.3	-	anoestrus		+/+		2	1	3	0	0	2
Mixed 4.2, 18.8	1.0	CLr	anoestrus		+/+		1	1	3	2	1	3
Mixed 6.3, 17.0	0.4	-	anoestrus		+/+		3	0	2	3	1	3
Anoestrus average: 4.6 years, 20.9 kg	0.8						1.9	1.3	2.8	1.1	1.0	2.4
Australian Terrier 2.4, 7.2	72.6	CL	dioestrus		+/+		2	0	2	3	2	3
Finnish Lapphund, 3.4, 17.6	n/a	CL	dioestrus	Missmated, non-gravid	+/+		2	3	3	3	3	3
Finnish Spitz 4.8, 11.7	6.1	CL	dioestrus		+/+	+	0	2	1	2	2	2
Mixed 0.8, 22.5	128.0	CL	dioestrus		+/+		3	3	3	0	2	2
Mixed 0.9, 39.0	8.2	CL	dioestrus		+++		0	2	2	2	2	3
Norwich Terrier 8.5, 6.3	27.3	CL	dioestrus	Uterine fibrosis	+/+		1	2	2	2	1	3
Portuguese Podengo 5.5, 5.1	163.3	CL	dioestrus	Missmated, non-gravid	+/+		2	3	3	2	2	2
Welsh Corgi Pembroke 2.2, 14.0	106.4	CL	dioestrus		+++		1	2	2	2	3	3
Dioestrus average: 3.6 years, 15.4 kg	73.1						1.4	2.1	2.3	2.0	2.1	2.6

Table 3. The individual and average data of 58 bitches divided in six groups in Study 2. To be continued.

Group	Mean age, min-max (years)	Mean weight, min-max (kg)	Mean litter size, min-max	Breed	Number of parturi-tions	Gestation length (days from the last and first mating)	Gestation length (days from ovulation)	Duration of the first stage of parturition (hours)	Duration of the second stage of parturition before intervention (hours), discharge	Litter size	Number of puppies born before dystocia / by Caesarean section	0: no medication 1: Ca and OT after blood sample
1. Complete primary uterine inertia (CUI) n = 7	4.1, 2.3-6.2	23.7, 4.7-35.0	6.4, 1.0-14.0	Border Collie	1	56-61	n/a	26	3, fetal fluids	4	0/4	1
				Airedale Terrier	1	60-62	63	45	6, fetal fluids	7	0/7	0
				Karelian Bear Dog	3	68	n/a	n/a	n/a, green fluids	1	0/1	0
				Dogo Argentino	1	65	n/a	29	6, green fluids	2	0/2	0
				C. Crested Dog	1	62	n/a	16	4, fetal fluids	6	0/6	1
				Engl. Spr. Spaniel	2	60	n/a	48	6, fetal fluids	14	0/14	1
				Labrador	1	59	60	65	6, fetal fluids	11	0/11	0
2. Partial primary uterine inertia (PUI) n = 13	5.1, 2.6-8.0	29.2, 6.5-65.0	7.0, 5.0-15.0	Mixed	1	64-65	n/a	n/a	4	5	1/4	1
				Labrador	2	60-62	62	5	4	8	5/3	1
				Dalmatian	3	63	63	6	4	6	1/5	0
				Poodle, Standard	1	61-62	62-63	7	9.5	5	3/2	0
				Labrador	2	63	n/a	10	5	8	6/2	1
				Min. Schnauzer	1	60-61	n/a	24	3.5	7	1/6	1
				Tibetan mastiff	2	59-61	62	24	4	5	4/1	1
				Mixed	1	62	n/a	27	n/a	7	1/6	1
				Border Collie	1	57-61	n/a	22	5.5	6	4/2	1
				Bullmastiff	2	56-57	57	65	n/a, fetal fluids	15	1/14	0
				Great Dane	1	62	n/a	26	6	6	2/4	1
				Bearded Collie	1	54-56	58	14	5.5	8	4/4	1
3. Obstructive dystocia (OD) n = 10	5.1, 2.3-8.3	23.7, 7.0-65.0	5.0, 1.0-9.0	Giant Schnauzer	2	58	60	12	10	5	2/3	1
				Cairn Terrier	3	64	66	n/a	4	1	0/1	0
				Border Collie	3	59-63	63	n/a	n/a, green fluids	7	0/7	1
				Saluki	2	61	64	18	4	3	0/3	0
				Cavalier	1	59-60	62	24	4	7	0/7	0
				French Bulldog	1	61-63	n/a	9	6	3	0/3	0
				Dog de Bordeaux	2	65-67	n/a	11	4	9	0/9	1
				Smooth Collie	3	60-61	n/a	24	8	6	3/3	1
				Cavalier	1	56-58	59	n/a	3	4	1/3	1
				Spanish Mastiff	1	62	62	11	n/a, fetal fluids	3	0/3	1
				Border Collie	3	60-61	61	24	3	7	0/7	1

Parity includes the current parturition. Medication: 0, no medication; 1, calcium and oxytocin after blood sample; n/a, not available.

Table 3. Continued. The individual and average data of 58 bitches divided in six groups in Study 2.

Group	Mean age, min-max (years)	Mean weight, min-max (kg)	Mean litter size, min-max	Breed	Number of parturi-tions	Gestation length (days from the last and first mating)	Gestation length (days from ovulation)	Duration of the first stage of parturition (hours)	Duration of the second stage of parturition before intervention (hours), discharge	Litter size	Number of puppies born before dystocia / by Caesarean section	0: no medication 1: Ca and OXT after blood sample
4. Elective caesarean section (ECS) n = 11	4.6, 3.1-6.6	23.9, 6.9-65.0	3.8, 1.0-10.0	Cavalier	3	n/a	60	0	0	4	0/4	0
				Border Collie	1	64-65	64	0	0	2	0/2	0
				St. Bernard	2	n/a	61	0	0	2	0/2	0
				Cavalier	3	58-60	60	0	0	5	0/5	0
				Great Dane	2	62-63	62-63	0	0	1	0/1	0
				Shetl. Sheepdog	4	60	n/a	0	0	1	0/1	0
				Boston Terrier	2	63-63	62	0	0	2	0/2	0
				Newfoundlander	1	58-64	n/a	0	0	10	0/10	0
				Cavalier	3	59-60	59	0	0	8	0/8	0
				Boston Terrier	1	63-65	n/a	0	0	3	0/3	0
				Golden Retriever	1	64-66	65	0	0	4	0/4	0
5. Medically treated dystocia, no caesarean section (MD) n = 8	4.7, 2.6-6.5	13.3, 4.5-20.0	5.1, 2.0-8.0	Dachshund	1	58-60	n/a	1	2	4	0/-	1
				Dwarf Dachshund	1	63	n/a	15	5	6	0/-	1
				Finnish Lapphund	1	n/a	n/a	n/a	7	5	4/-	1
				Jack Russel Terrier	2	58-61	n/a	n/a	4	2	0/-	1
				Australian Kelpie	1	60	n/a	15	7	8	6/-	1
				Engl. Spr. Spaniel	3	n/a	n/a	12	10	4	2/-	1
				Finnish Lapphund	2	n/a	n/a	n/a	5	6	3/-	1
				Lapponian Herder	2	n/a	63	7	5	6	2/-	1
				Cavalier	1	n/a	60	-	-	5	-	0
				Labrador	3	n/a	60	-	-	10	-	0
6. Control, normal parturition (C) n = 9	4.20, 1.9-6.5	10.9, 8.0-30.0	5.4, 4.0-10.0	Cavalier	4	n/a	59	-	-	4	-	0
				Pug	2	n/a	61-62	-	-	5	-	0
				Pug	1	n/a	61	-	-	5	-	0
				Cavalier	4	61	n/a	-	-	5	-	0
				Cavalier	1	62	n/a	-	-	4	-	0
				Cavalier	2	n/a	58-59	-	-	5	-	0
				Cavalier	2	n/a	60	-	-	6	-	0

Parity includes the current parturition. Medication: 0, no medication; 1, calcium and oxytocin after blood sample; n/a, not available.

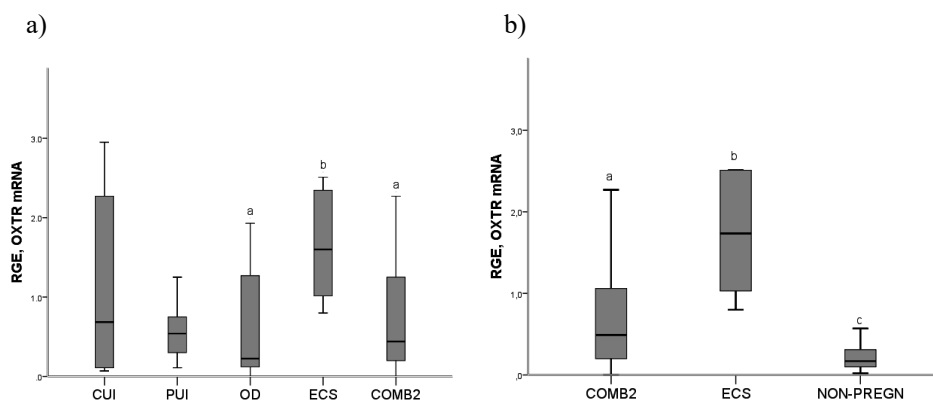


Figure 6. Relative gene expression (RGE) of canine uterine oxytocin receptor (OXTR) mRNA; a) prepartum (ECS) and dystocic bitches (Study II), b) prepartum (ECS), dystocic, and non-pregnant bitches (unpublished data). The boxplots show the median with 50% of the data falling within the box. The whiskers extend to the 5th and 95th percentiles. Boxes with different letter designations are significantly different at $p < 0.05$. CUI, complete uterine inertia; PUI, partial uterine inertia; OD, obstructive dystocia; ECS, elective Caesarean section; COMB2, three dystocia groups combined: CUI, PUI, OD; NON-PREGN, non-pregnant bitches.

5.2.3 Oxytocin receptors, IHC (I, II)

The staining for OXTR in IHC was observed in the cytoplasm of cells in myometrium (MYO), endometrial luminal epithelium (LE) and endometrial deep and superficial glandular epithelium (GE) (Fig. 7; Fig. 8). A weak staining was also noticed in the endometrial stroma. Leucocytes and endothelium of blood vessels were positively stained in all samples, with the exception of one uterine body sample (Study I) in which no leucocytes were detected. No difference in staining intensity in MYO, LE or GE was detected in Study I between the horn and the body. A significant difference was noticed in the LE between anoestrous and dioestrous uteri: The staining intensity was higher in dioestrus than in anoestrus ($p < 0.05$). No difference was noticed in MYO or GE between anoestrous and dioestrous uteri, or between circular and longitudinal layers of MYO. There was no difference in Study II in staining intensity between the dystocia groups. The staining intensity in MYO was significantly higher in pregnant bitches, in both COMB2 and ECS, than in non-pregnant ones (Fig. 9). In GE the staining intensity was significantly higher in non-pregnant bitches than in pregnant ones (COMB2 and ECS) but in LE no difference was noted (Fig. 9).

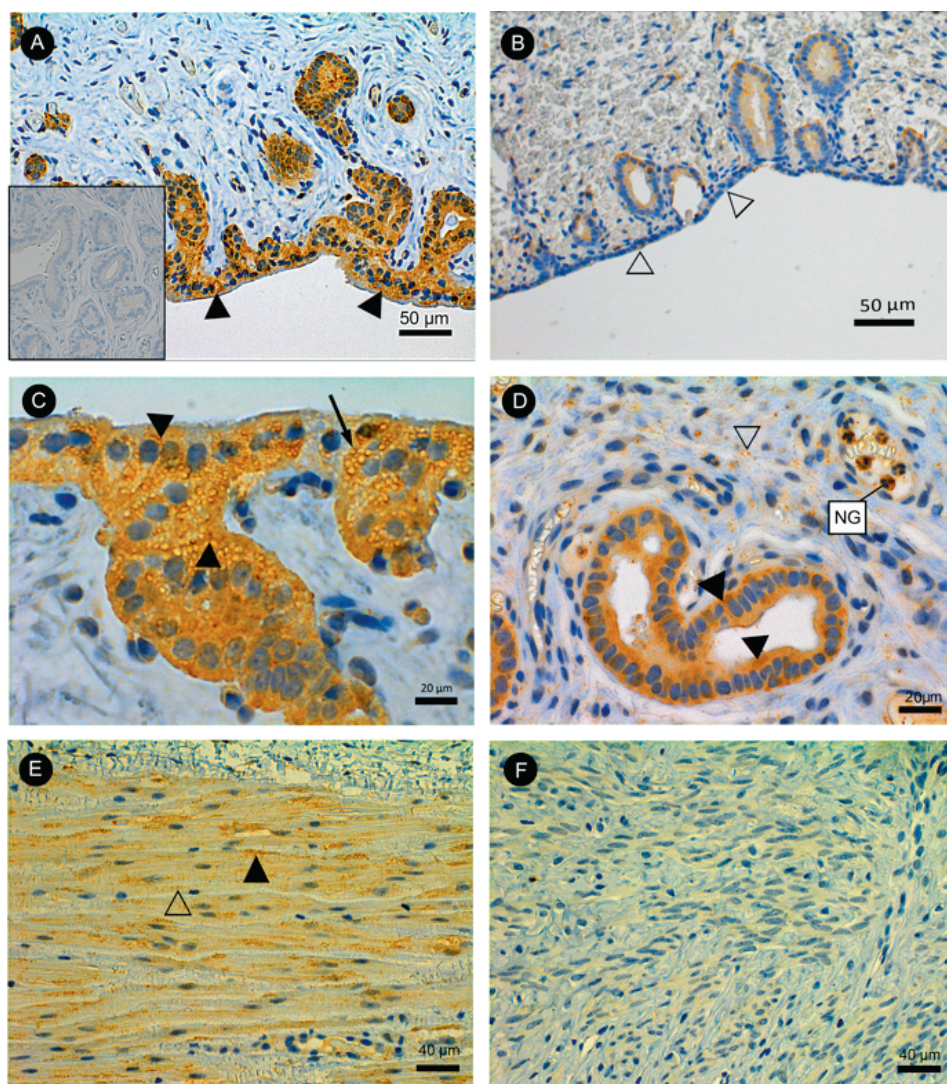


Figure 7. Immunohistochemical staining for oxytocin receptor (OXTR) in the non-pregnant canine uterus. Brown colour indicates positive staining for OXTR. A) Dioestrus, middle horn, endometrial luminal epithelium: strong signal (solid arrowhead). Insert: negative control, no background staining. B) Anoestrus, middle horn, endometrial luminal epithelium: very weak signal (open arrowhead). C) Dioestrus, middle horn, degenerating endometrial luminal epithelium: strong signal (solid arrowhead), vacuoles (arrow). D) Dioestrus, body, endometrium, glandular epithelium of superficial gland: strong signal (solid arrowhead) and stroma: weak signal (open arrowhead), neutrophil granulocyte (NG): strong signal. E) Dioestrus, body, myometrium, circular layer: strong (solid arrowhead) and weak (open arrowhead) signals. F) Dioestrus, body, myometrium, longitudinal layer, negative control: no background staining. (Study II)

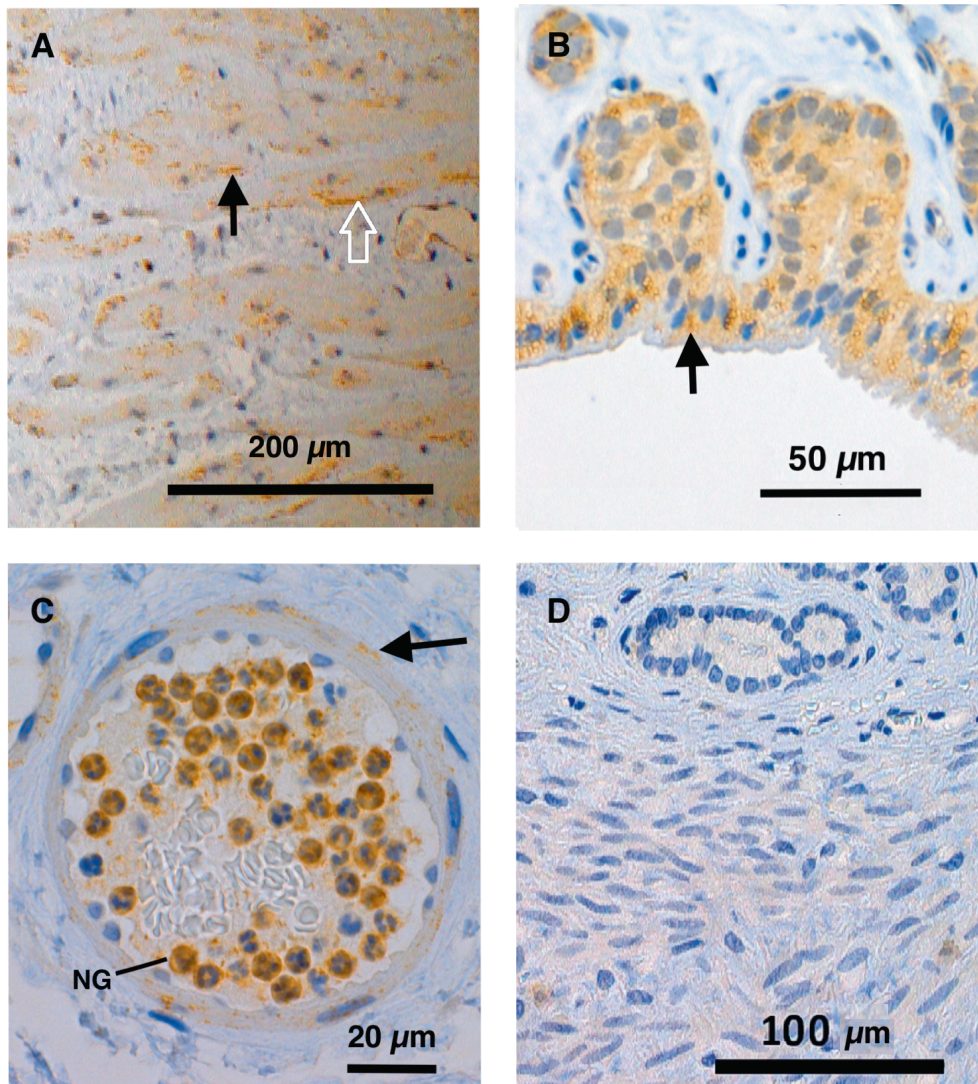


Figure 8. Immunohistochemical staining for the oxytocin receptor (OXTR) in the pregnant canine uterus. A brown colour indicates positive staining for OXTR. A) Moderate (black arrow) and strong (arrow with white outline) signal of myometrium. B) Moderate signal of endometrial luminal epithelium (black arrow). C) Weak signal of endothelium (black arrow) and strong signal of neutrophil granulocyte (NG). D) Negative control for background staining (myometrium, endometrial glands). Unpublished data.

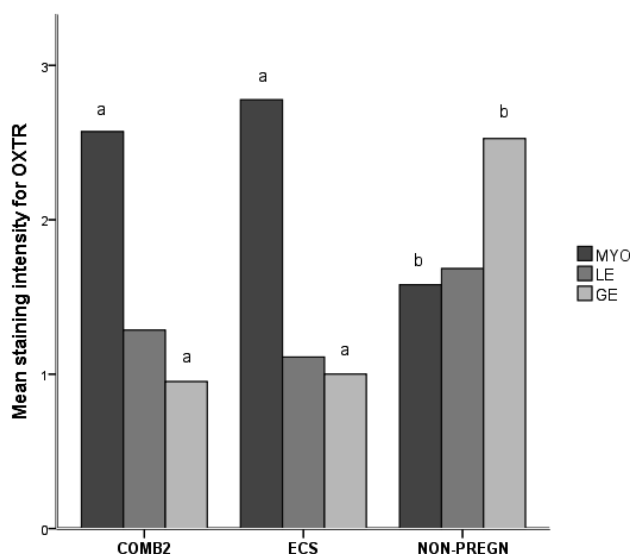


Figure 9. Mean staining intensity for oxytocin receptor (OXTR) in immunohistochemistry. COMB2, three dystocia groups combined: complete uterine inertia, partial uterine inertia and obstructive dystocia; ECS, elective Caesarean section; NON-PREGN, non-pregnant; MYO, myometrium, LE, endometrial luminal epithelium, GE, endometrial glandular epithelium. Bars with different letter designations in each tissue group are significantly different at $p < 0.05$. Unpublished data.

5.3 Cardiotocography (III)

Parturition was monitored measuring uterine contractions and foetal heartbeats with cardiotocography in pregnant bitches before and during parturition. Figure 10 demonstrates the positioning of the tocographic probe and Table 4 describes the individuals of the study. Figure 11 presents examples of different contraction patterns; Table 5 presents observed contractions. The three CUI bitches showed no contractions. The relative change of pressure (amplitude change) was lowest in PRE (10–15% units) and highest in NORM (20–45% units), and a significant difference ($p < 0.05$) was noticed between PRE and NORM, PRE and OD, and PRE and MD. The number of contractions in 10 minutes was lowest in PRE (0.7) and highest in OD (1.3) and MD (1.3), and a significant difference was detected between PRE and NORM, PRE and PUI, and PRE and MD. The mean duration of contractions was shortest in PRE (2.1 min) and longest in NORM (3.1 min), and a significant difference was discovered between PRE and NORM and PRE and MD. The total number of contractions and the interval between contractions were not compared due to the great variation in monitoring times. In PUI, 6 bitches of 8 were treated with oxytocin before CS: A weak

response (10–25% units amplitude change) was noticed in 5 bitches and 1 bitch did not respond (no amplitude change). In MD, all 6 bitches were treated with oxytocin: A strong response (25–45% units amplitude change) was noticed in 5 bitches and in 1 bitch the response was weak (15% units amplitude change), but this bitch gave birth (6 puppies) without CS.

Panting and movements of a bitch resulted in short-lasting, sharp spikes in graphs (Fig. 11d). These spikes were also noticed in non-pregnant individuals when they moved (amplitude change 10–20% units) but panting caused even higher spikes (amplitude change up to 90% units). A bitch lying on the probe for a while resulted in a similar pattern as in uterine contraction but with a higher amplitude change (40–70% units). The monitoring was impossible in very restless bitches. Four bitches were clearly uncomfortable with the probe and the belt around their abdomen; the monitoring was discontinued in those cases. Two bitches tried to bite the belt even after the position of the probe was corrected and the belt was loosened.

Foetal heartbeats were detected in 85% of cases. Heartbeats were easier to recognise individually when the litter size was less than 4, whereas it was impossible in large litters. The most common factors for erroneous results were incorrect positioning of the probe, restless movements and the dam's overweight. The probe readily recorded the maternal heartbeat instead of the foetal. For clinical decisions, the foetal heart rate was ensured by ultrasonographic examination.



Figure 10. Positioning of the tocographic probe for detecting uterine contractions using TeamCare CTG monitor. (Study III)

Table 4. The individual data of 47 bitches divided in 7 groups in Study III.

	Breed	Age (years)	Weight (kg)	Litter size	Gestation length from ovulation during monitoring	Gestation length from last mating	Monitoring sessions	OXT
PRE n = 13	New Foundland	3	50	10	n/a	62	1	0
	Golden Retriever	4	28	4	61	n/a	1	0
	CKCS	3	8	6	55	n/a	1	0
	CKCS	3	9	6	56	n/a	1	0
	Podengo Portuguese	4	5	3	n/a	61	1	0
	Jack Russel Terrier	2	6	1	n/a	61	1	0
	CKCS	4	10	8	59	59	1	0
	Miniature Poodle ^a	6	7	3	57, 61	n/a	5	0
	Pug ^b	3	8	5	60	n/a	1	0
	Lapponian Herder ^c	5	17	6	58, 60, 61	n/a	3	0
	Labrador Retriever	5	28	6	n/a	55, 61	2	0
	CKCS	3	7	4	61	n/a	1	0
	CKCS	4	10	4	60	n/a	1	0
FIRST n = 7	Border Collie ^d	4	15	6	n/a	57, 61	2	0
	Pug ^b	3	8	5	61, 62	n/a	2	0
	Lapponian Herder ^c	5	17	6	62	n/a	5	0
	CKCS ^c	4	8	4	n/a	62	2	0
	CKCS	3	8	6	60	n/a	1	0
	Miniature Poodle ^a	4	7	2	61	n/a	1	0
	CKCS	4	10	4	62	n/a	1	0
NORM n = 17	Dobermann	4	34	9	62	n/a	3	0
	New Foundland	4	52	9	63	62	2	0
	Labrador Retriever	5	30	10	60	n/a	3	0
	CKCS	3	10	5	60	59	1	0
	CKCS	3	8	4	59	58	1	0
	Pug ^b	3	8	5	61, 62	n/a	4	0
	Pug	4	8	5	61	n/a	5	0
	CKCS	2	9	5	n/a	61	2	0
	CKCS ^c	4	8	4	n/a	62	4	0
	CKCS	6	10	5	59	n/a	1	0
	CKCS	3	8	6	62	n/a	1	0
	Miniature Poodle ^a	4	7	2	62	n/a	1	0
	Giant Schnauzer	5	32	7	n/a	62	5	0
	CKCS	6	9	6	61	n/a	1	0
	CKCS	4	7	5	58	n/a	1	0
	Giant Schnauzer	6	28	10	n/a	62	3	0
	Giant Schnauzer	4	30	11	n/a	60	5	0
CUI n = 3	Border Collie	4	16	4	n/a	61	2	0
	Airedale Terrier	5	23	7	63	60	1	0
	Labrador Retriever	3	35	11	63	60	1	0
PUI n = 8	Labrador Retriever	3	30	8	62	60	1	1
	Border Collie ^d	4	15	6	n/a	58	1	1
	Bullmastiff	3	52	15	60	59	1	0
	Great Dane	3	65	6	n/a	62	1	1
	Labrador Retriever	4	30	8	n/a	63	1	1
	Tibetan Mastiff	3	40	5	n/a	63	2	1
	Mixed	4	40	7	n/a	62	2	1
OD n = 3	Giant Schnauzer	5	32	4	62	n/a	2	0
	Border Collie	6	18	6	63	59	1	0
	CKCS	4	7	4	59	57	2	0
MD n = 6	Spanish Mastiff	3	65	3	63	n/a	1	0
	Finnish Lapphund	4	15	5	n/a	62	1	1
	Australian Kelpie	5	17	8	n/a	60	2	1
	Engl.Springer Spaniel	4	20	4	n/a	61	1	1
	Finnish Lapphund	4	18	6	n/a	60	1	1
	Pug ^b	3	8	5	61	n/a	1	1
	Lapponian Herder ^c	5	17	6	63	n/a	2	1

PRE, prepartum; FIRST, the first stage of parturition; NORM, the second stage of normal parturition; CUI, complete uterine inertia; PUI, partial uterine inertia; OD, obstructive dystocia; MD, medically treated dystocia; CKCS, Cavalier King Charles Spaniel; OXT, oxytocin treatment (0, no treatment, 1, OXT treatment). Same letter after breed indicates same individual in different groups.

Table 5. Observed uterine contractions during cardiotocography monitoring in different stages of parturition in 47 pregnant bitches in Study III. PRE, prepartum, FIRST, the first stage of parturition; NORM, the second stage of normal parturition; CUI, complete uterine inertia; PUI, partial uterine inertia; OD, obstructive dystocia; MD, medically treated dystocia. Letters in superscript indicate a significant difference ($p < 0.05$) within the column.

Group		Individual monitoring time, min (mean)	Number of contractions (mean)	Mean number of contractions /10 min	Change of amplitude %-units (mean)	Interval between contractions, min (mean)	Duration of contractions, min (mean)
Prepartum (PRE, n=13)		8-170 (39)	0-3 (2.0)	0.7 ^a	10-15 ^a (13.8)	4-9 (6.5)	1-3 (2.1) ^a
First stage of parturition (FIRST, n=7)		7-165 (57)	0-11 (3.9)	0.9	10-30 (21.7)	4-21 (10.6)	1-4 (2.6)
Second stage of parturition (n=37)	NORM (n=17)	13-140 (86)	1-31 (8.3)	1.1 ^b	20-45 ^b (32.6)	1-14 (6.2)	2-5 (3.1) ^b
	CUI (n=3)	20-45 (32)	0	0	<10	-	<1
	PUI (n=8)	10-110 (42)	1-14 (5.0)	1.2 ^b	10-30 (17.5)	4-13 (7.3)	1-4 (2.3)
	OD (n=3)	20-45 (29)	3-4 (3.3)	1.3	20-40 ^b (30.0)	2-8 (5.0)	2-4 (3.0)
	MD (n=6)	14-56 (34)	1-7 (4.2)	1.3 ^b	15-45 ^b (29.1)	3-4 (3.5)	2-4.5 (3.0) ^b

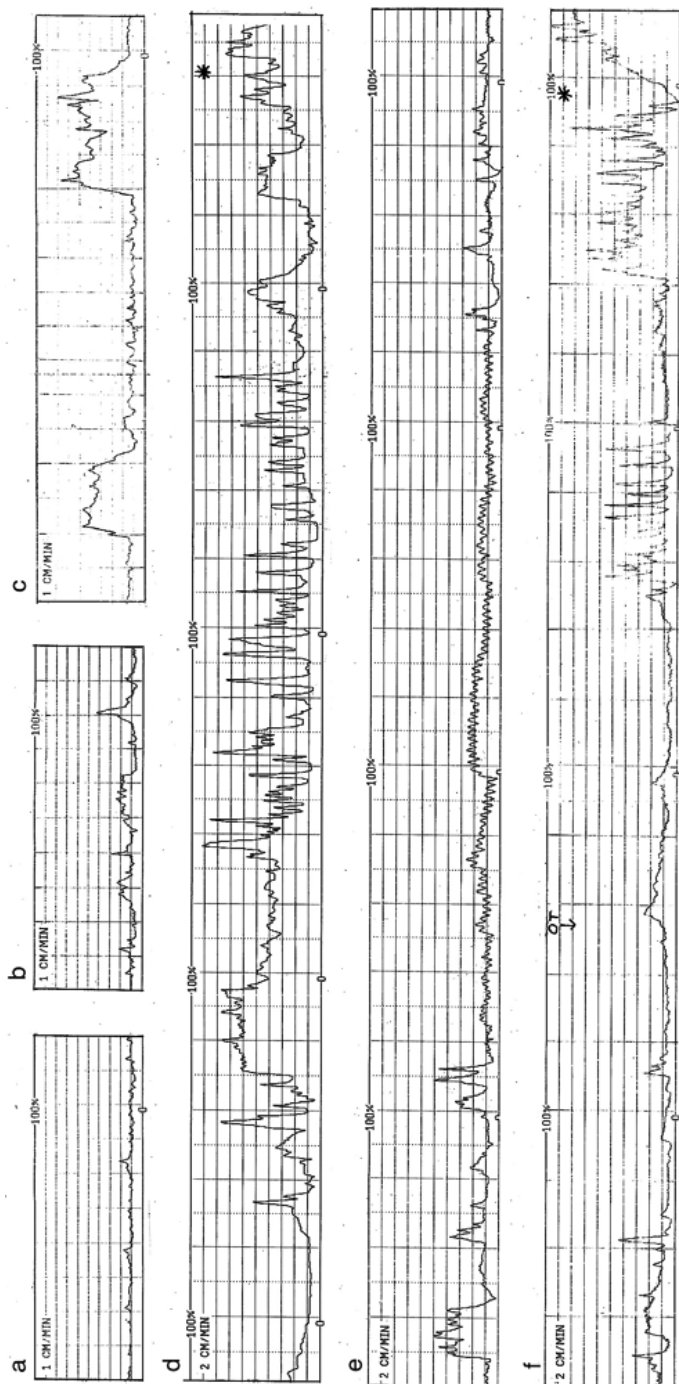


Figure 11. Examples of uterine activity presented as tocographs of relative pressure change (%-units): a) prepartum, no contractions; b) first stage of parturition, same bitch as in (a) but two days later, no clear contractions but change in uterine activity, first puppy was born 5 hours later; c) the second stage of normal parturition, peaceful dam, two contractions; d) the second stage of normal parturition, restlessly panting dam, one clear contraction, strong abdominal straining, puppy birth marked with asterisk; e) uterine inertia, same bitch as in (d), 4 hours later, no response to medical treatment, ended up in Caesarean section; f) uterine inertia, 12 minutes after oxytocin administration (marked with OT and arrow) last puppy was born (marked with asterisk), abdominal straining and one clear contraction. The scale of the original graph is 1 cm/min in a-c, and 2 cm/min in d-f. (Study III)

6 Discussion

6.1 The uterus of the non-pregnant bitch

The expression of OXTR in the uterus of non-pregnant bitches during anoestrus and dioestrus supports the assumption that OXT has a role in contractile activity not only during parturition but also in anoestrus and dioestrus phases. Ibuki et al. (1997) demonstrated uterine contractions in non-pregnant bitches during anoestrus and dioestrus using an implanted uterine force transducer, and administration of OXT stimulated the contractile activity. Also, *in vitro* studies in myometrial fibres from anoestrous and dioestrous bitches showed that myometrium in these stages is capable of responding by contraction to OXT and natural PGF2 α (Gogny et al. 2010).

Derussi et al. (2012) reported the expression of OXTR in the middle horn of the non-pregnant bitch. We saw the expression of OXTR also in the body in addition to the middle horn. The staining intensity in the LE was significantly stronger in dioestrous than in anoestrous uteri. The uterus eliminates possible infection after uterine bacterial invasion through the cervical opening in oestrus. Local endometrial paracrine or autocrine signalling of the OXT and OXTR systems may have a role in this cleaning process and may, thus, explain the higher staining intensity of OXTR in LE in dioestrus than in anoestrus.

The uterine paracrine or autocrine signalling may also be involved in embryo implantation. Unlike in many other species, endometrial expression of OXTR in the canine uterus probably has no influence on luteolysis, because it has been shown that hysterectomy does not extend the luteal phase (Hoffman et al. 1992). The presence of OXTR in the uterus of a non-pregnant bitch may also be considered as a standby mode, or the readiness of the uterus for changing to more intense contractions.

The neutrophil granulocytes were positively stained for OXTR in our study. This has not been reported earlier, to our knowledge. However, the expression of OXTR in the neutrophil granulocytes may have been unspecific and, therefore, should be further verified, for example, by Western Blotting analysis. Bovine T lymphocytes express OXTR, and E2 and P4 play a role in the regulation of this expression (Ndiaye et al. 2008). Some evidence in rats shows that OXT decreases inflammatory processes, possibly by antioxidant action, increased endogenous glucocorticoids and diminished neutrophil infiltration (Petersson et al. 2001; Iseri et al. 2005; Biyikli et al. 2006), but the role in the canine uterus is unclear.

Histological differences between this study's anoestrous and dioestrous uterine samples resemble those previously reported (Galabova et al. 2003; Rehm et al. 2007). The endometrium collapses in late dioestrus: The cytoplasm of endometrial luminal epithelial and crypt

cells is vacuolated (Rehm et al. 2007). Nuclear density seems higher due to atrophic changes in anoestrus: Less cytoplasm is seen in anoestrous uterine samples (Rehm et al. 2007). In both groups, anoestrus and dioestrus, two bitches showed occasionally leucocytes in several fields (++) or several in all fields (+++). This may indicate that these individuals had subclinical endometritis, which may alter the functionality of the endometrium and expression of OXTR.

6.2 Regulation of oxytocin receptors in bitches

The peripheral blood hormone concentrations are similar to non-pregnant bitches in dioestrus during the first trimester of pregnancy (Concannon et al. 1975; Concannon et al. 1989; Onclin et al. 2002). LH, FSH (follicle stimulating hormone) (Onclin et al. 2002), relaxin (Tsutsui and Stewart 1991), and prolactin (Concannon et al. 1978) levels are higher in pregnant bitches during the last two-thirds of pregnancy than in non-pregnant bitches in dioestrus. The P4 levels are similar in pregnant and non-pregnant bitches, except for the more sudden drop in the pregnant ones before parturition (Concannon et al. 1975). The E2 concentrations have large individual variation, but the levels are similarly elevated during dioestrus and pregnancy (Onclin et al. 2002).

The OXTR expression in the uterus of dystocic bitches has not, to our knowledge, been published earlier. Also, other OXTR studies in dogs are sparse, but it has been shown that the expression of OXTR is higher in the uterus of pregnant bitches near term compared with earlier stages of pregnancy, which indicates that OXTRs are upregulated at the time of the prepartum luteolysis (Derussi et al. 2012; Gram et al. 2014; Veiga et al. 2015). In our study, the relative expression of OXTR was highest prior to the initiation of parturition (ECS group). Overall, the expression was higher in the uterus of prepartum and dystocic bitches than in dioestrous or anoestrous bitches. We did not investigate bitches during earlier stages of pregnancy.

In *in vitro* studies with myometrial fibres, the response to OXT was increased in dioestrous bitches treated once with an antigestagen injection 3–5 days before OHE, which supports the assumption of P4 blocking the contractile activity of the myometrium (Gogny et al. 2010). OXTR was upregulated in normal and aglepristone-induced parturition (Gram et al. 2014). The absence of P4 as such cannot be the only stimulation for OXTR expression, because in our study the anoestrous basal level of P4 did not upregulate the OXTR.

Our results support previous reports on decreasing P4 (Concannon et al. 1975; Concannon et al. 1988; Nohr et al. 1993; Hoffmann et al. 1992; Veronesi et al. 2002) and increasing PGFM (Concannon et al. 1988; Nohr et al. 1993; Veronesi et al. 2002) levels during prepartum luteolysis in pregnant bitches. As expected, P4 levels were higher in the ECS group than in the CUI, PUI, OD, MD, and C groups, as CS was performed in this group before the onset of parturition (before stage 1). Interestingly, in the ECS group the relative expression of OXTR was high even

when the P4 levels were still high. The decrease of P4 and increase of E2 is considered to regulate OXTR upregulation in many species such as cow (Fuchs et al. 1992), pig (Lundin-Schiller et al. 1996), and sheep (Beard and Lamming 1994; Wathes et al. 1996). In dogs, the gradual decrease of P4 is more likely involved in OXTR upregulation, because the increase in relative expression seems to occur before the sudden decline of P4. Termination of luteal function in non-pregnant bitches is more likely regressive than the active luteolytic process found in pregnant bitches, which suggests a different regulation mechanism (Kowalewski 2014a). Failure of luteolysis can lead to prolonged gestation (Irons et al. 1997). All bitches except in the ECS group had undergone luteolysis in this study.

Expression of the P4 receptor mRNA is highest in the canine uterus during the pre-implantation period and lower during the rest of the gestation (Kowalewski et al. 2010, 2014b). This ensures the survival of the embryos, because the responsiveness of the endometrial epithelium to P4 secures the uterine milk production (Kowalewski et al. 2010). P4 also has a role in preventing immunological rejection of the embryos in utero in rats (Ragusa et al. 2004). The suppression of the P4 receptor function, not the expression, is considered to increase OXTR expression in decidual cells as part of foeto-maternal signalling and prepartum release of placental PGF2 α (Kowalewski et al. 2010).

We could not use the results of the E2 analysis, because in most samples the levels were below detection limit. The method seemed to be acceptable according to the standard curves and a control sample from an oestrous bitch with a high E2 level. The bitch has no marked elevation of E2 associated with parturition as seen in many other species (rat: Yoshinaga et al. 1969; sheep: Robertson and Smeaton 1973; cow: Robertson 1974). Oestrogens increase uterine OXTR expression in many species (Richard and Zingg 1990; Beard and Lamming 1994; Zingg and Laporte 2003). E2 is at a slightly elevated level in the last trimester of pregnancy in the bitch and decreases suddenly 2 days prior to parturition (Concannon et al. 1975; Hoffmann et al. 1994; Onclin et al. 2002); high E2 levels cannot, thus, explain the prepartum increase of OXTR. It is obvious that the low levels of E2 in periparturient bitches were actual in our study. The bitches in the ECS group were near term, and the levels of E2 were probably truly low. The questions arise: is the OXTR upregulated by the earlier moderate increase in E2, is the decrease of E2 involved in the upregulation, or does the upregulation occur regardless of E2 changes?

6.3 Uterine inertia

The aetiology of uterine inertia is multifactorial. Any reason that prolongs the parturition may disturb the contractility of the uterus. Indeed, a large proportion of dystocia cases that are classified

as uterine inertia may be secondarily due to several factors that prolong the parturition and lead to uterine fatigue.

Our study suggests that in CUI the aetiology is not the absence or downregulation of OXTR. There was no difference in OXTR expression between CUI and bitches near term but before the first stage of parturition (ECS group). Upregulation of OXTR occurs near term, and the prolonged influence of OXT and uterine exhaustion in PUI and OD may lead to downregulation of OXTR as in other species (Engström et al. 1988; Eiler et al. 1989; Phaneuf et al. 1997).

One possibility for the aetiology of CUI could be a problem in parturition initiation. Excessive P4 and insufficient PGF2 α levels could prevent uterine contractions, and thus, interfere with parturition. However, our results suggest that this may not be the case, because the P4 and PGFM levels in CUI group were similar to other dystocia groups.

Uterine stretching increases OXTR expression in the rat uterus (Ou et al. 1998) and alters the calcium signalling in human myometrium by increasing calcium entry into myometrial cells (Dalrymple et al. 2007). In dogs, a lack of stretching of the uterus in case of very small litters may affect OXTR expression and calcium signalling and, thus, the contractility of the uterus, which may lead to CUI. There were only two small litters in the CUI group in our study, and no further conclusion can be made.

A high P4:PGFM ratio has been reported in dystocic bitches with CUI in comparison to a control group (Bergström et al. 2010). No such difference was observed in our study. This may indicate that the aetiology of CUI is more likely at the level of uterine function, such as myometrial distention beyond its capacity to contract or the lack of cervical pressure to stimulate OXT release. Abnormal foetal hypothalamo-pituitary-adrenocortical function could be one reason for CUI in cases where gestation is overdue, and placentas begin to detach.

OXTRs were present in vascular endothelial cells as previously reported in the canine (Gram et al. 2014) and ovine (Wu et al. 1996) uterus. OXT has been reported to cause relaxation or contractions depending on the type of the blood vessel in dogs (Katusic et al. 1986). The purpose of OXT actions in utero-placental vasculature is unclear, but it may be important in placental detachment (Weeks 2008) and in closure of the umbilical vessels at birth (Altura and Altura 1984).

Uterine contractions are easily detected with the CTG, and hence, diagnosis of uterine inertia can be confirmed and the response to uterotonic medicines followed. No contractions were detected in CUI group bitches. There was no difference in contractility (mean number of contractions in 10 min, amplitude change, duration of the contraction) between PUI and NORM or between MD and NORM. However, we noted that previously strong contractions ceased in PUI and MD, and there was a mild response to OXT treatment in PUI that did not lead to puppy expel, but in MD the response was strong, resulting in the birth of a puppy.

Calcium and OXT injections or infusions are used as a treatment of uterine inertia to enhance uterine contractions (Johnston et al. 2001; Davidson 2010). Batra (1986) reported that OXT-induced myometrial contractions in the rat depend on the influx of extracellular calcium, and this influx is directly increased by OXT. In humans, OXT has been postulated to inhibit the Ca^{2+} -extrusion pump (Popescu et al. 1985). Hypocalcaemia was not diagnosed in any of the bitches in this study. However, there are reports of hypocalcaemia in risk groups of uterine inertia (Hollinshead et al. 2010) and in dystocic bitches (Frehner et al 2018). In our study, a single treatment with calcium gluconate and OXT did not seem to affect the expression of OXTR mRNA or distribution of OXTR. However, the uterine samples were not repeated samples from the same bitches as before and after the treatment; the comparison was made between the treated and untreated individuals. Prolonged exposure to OXT downregulates myometrial OXTR in rabbits (Eiler et al. 1989), rats (Engström et al. 1988) and humans (Phaneuf et al. 1997). The uterus has been under the influence of OXT in dystocic bitches, and exhaustion and desensitization may prevent the effect of medicines to enhance uterine contractions. However, in this study the number of bitches treated or not treated was low, and further investigation is necessary.

The clinical diagnosis of uterine inertia is based on visible abdominal contractions, the behaviour of the bitch, the progress of the parturition, and clinical examination of the bitch, including vaginal and ultrasonographic examination (Johnston et al. 2001; Linde-Forsberg 2010). When medical treatment is considered, it is crucial to exclude obstructive cause to avoid unnecessary pain, delaying the CS decision, and risk survival of the puppies. The contractility of the uterus can be confirmed using CTG. It helps also to assess whether the parturition has started or not.

6.4 Cardiotocography in canine parturition monitoring

Dystocia diagnosis may be challenging and sometimes it is difficult to define. The external parturition monitoring system has been used in the United States (Whelpwise™ Veterinary Perinatal Specialties®), but only a few reports exist about the method in dogs (Davidson 2001; Schröder et al. 2006; Groppetti et al. 2010), and interpretation guidelines for CTG monitoring in bitches do not exist. Setting clear limits and criteria for normal and abnormal contraction patterns in dogs may be difficult due to the great variety in the number of the puppies, in the bitches' size, and the bitches' behaviour during parturition.

Both monitors (Sonicaid FM 820, TeamCare) proved to be useful in parturition monitoring in this study. Uterine contractions are not externally visible during the first stage of parturition (Johnston et al. 2001; Linde-Forsberg 2010), but CTG detects these preparing contractions. In our study, we could not differentiate prepartum and first stage bitches according

to contraction numbers, amplitude changes, or duration of contractions. Prepartum bitches had significantly fewer contractions in 10 minutes in comparison to NORM, PUI, and MD, which shows that parturient bitches are easily distinguished from those whose parturition has not yet started. However, bitches with complete uterine inertia cannot be differentiated from prepartum or first stage bitches by using CTG only. Vaginal examination and visualization of an amniotic sac or foetal parts should be performed to confirm whether the bitch is in labour or not. No significant difference was noted in the average number of contractions in 10 minutes, in change of amplitude, or in duration of contractions between prepartum and the first stage bitches. This may be due to a too short monitoring time; some contractions may have been missed.

It seems that uterine contractions in the bitch commonly last longer than two minutes during normal parturition. The contractions lasted significantly longer in the second stage NORM and MD groups than in the PRE group. In women, periparturient uterine contractions usually last from 45 to 120 seconds (Ayres-de-Campos et al. 2015). More than five contractions in 10 minutes during two 10-min periods or averaged over a 30-min period are considered in women as a tachysystole, excessive frequency of uterine contractions (Macones et al. 2008; Ayres-de-Campos et al. 2015). Tachysystole has not, to our knowledge, been described in dogs. This does not mean that tachysystole does not exist in dogs. It has not been described by now and, consequently, definition criteria do not exist. None of the bitches in our study had more than five contractions in 10 minutes. The number of contractions in 10 minutes is probably not the optimal way to describe contractility, because bitches usually have multiple offspring, and the resting periods between the delivery of puppies may be long (Johnston et al. 2001; Linde-Forsberg 2010).

Foetal heartbeats were detected with CTG, but the method is sensitive to artefacts, and maternal heartbeats were easily recorded instead of foetal ones, which is also a recognized problem in humans (Nurani et al. 2012). The canine foetus and its heart are smaller, and its heart rate (160–220 bpm: Linde-Forsberg 2010) is faster in comparison to a human full-term foetus (110–160 bpm) (Ayres-de-Campos et al. 2015). These anatomical and physiological features of dogs may complicate the use of the method. Dogs usually have multiple offspring, and it is impossible to follow the heart rates of all foetuses. The caudal ones that are first presented to the birth canal are usually also the first ones in distress. Monitoring of foetal distress in dogs subsequent to uterine contraction would be of interest, because uterine contractions reduce the placental blood flow, decreasing oxygen supply to the foetus (Assali et al. 1958; Sato et al. 2016). We could not demonstrate foetal distress after uterine contractions with CTG in our study, but it was confirmed with ultrasound examination. It was difficult to assure the recognised heartbeat was exactly from the foetus of concern in many cases. It also takes time to localise the heartbeat with CTG; ultrasound is faster. The monitoring protocol needs improvement to advance the diagnostics of foetal distress in dogs. This may be achieved simply by improving the routine of heartbeat follow-up with CTG or routinely combining the CTG and US examinations.

In the clinical use of CTG, a systematic follow-up of individuals should be conducted. Ideally, monitoring should be started before the first stage of parturition to familiarize the bitch with the procedure and to obtain an individual basal curve to detect the progress in uterine contractile activity. This requires monitoring at home, because bringing a bitch unnecessarily to the clinic environment is inadvisable. Bitches with a high-risk pregnancy and parturition could be hospitalized. It is recommended that the clinical staff become well acquainted with the CTG device to be used to learn which paper speed provides the graphs easiest to interpret, what kind of artefacts to expect, and how to adjust the probe correctly. Hair clipping may help to keep the probe in place in bitches with a heavy coat, but clipping does not seem to be necessary to detect pressure changes. During long, even continuous monitoring, the precision of the measuring of the number of contractions could be increased. Parturition follow-up should be done in cooperation with the breeder and a fully trained animal hospital staff. The CTG should never be used alone in diagnosis. A complete history, thorough clinical examination and ultrasonographic evaluation of the viability of foetuses should be performed every time. The actual pregnancy is confirmed by palpation and ultrasonographic examination. Radiographic imaging may be necessary to detect abnormalities such as mummified, malformed, or oversized foetuses. An obstruction of the birth canal due to an oversized foetus or an abnormal presentation, position or posture can also be confirmed with radiographic examination. The ovulation or mating dates are checked carefully to count the duration of the pregnancy. If there is any confusion about gestation time, ultrasonographic examination is helpful for estimating the foetuses' developmental stage (Luvoni and Beccaglia 2006; Beccaglia and Luvoni 2012). A sudden prepartum decrease of peripheral blood P4 indicates that a bitch is in labour (Concannon et al. 1975). Therefore, P4 analysis can be used to determine whether the bitch is in labour or not. However, it should be taken into account that some bitches are ready to give birth in 12 hours even when the progesterone level is still 15.8 nmol/l or more (De Cramer and Nöthling 2018).

6.5 Limitations of the study

Although strict criteria were defined to include the bitches in the groups in this study, some heterogeneity exists. Breed diversity also increases the heterogeneity of the groups. However, heterogeneous groups give a more realistic view of the real population than using only one breed of laboratory dogs. The Cavalier King Charles Spaniel was the most common breed in studies II and III; the control group was mostly this breed in study II, which may bias the results. However, this breed was also well represented in OD and ECS groups in study II. Bitches in groups MD and C had a lower average body weight than in other groups in study II, which may skew the results, because miniature and small breeds may have a greater risk to develop dystocia (Gaudet 1985;

Bergström et al. 2006; Münnich and Küchenmeister 2009). However, small and miniature breeds were also represented in the other groups (CUI, PUI, OD, ECS), but very large breeds increased the average body weight in these groups. The bitches with normal parturition were used in this study as controls only for the blood parameters. For OXTR gene expression, uterine samples only from prepartum (ECS) and dystocic (CUI, PUI, OD) bitches were used. Further studies are necessary to compare uterine OXTR gene expression in dystocic bitches and bitches with normal parturition. The number of bitches was quite low (particularly in the CUI groups in Studies II and III), which may affect the results. A greater number of individuals is necessary to properly evaluate the effect of calcium and OXT treatment on OXTR. The antibody specificity was not verified in this study; thus, the results should be confirmed by Western Blotting method.

The monitoring time in the CTG study was quite short on many occasions; consequently, some contractions may have been missed. Shorter contractions, the ones below the one-minute limit – the study's setting – may also be relevant for the progress of the parturition. Asynchronous contractions in the two horns may also occur, which may lead to misinterpretations. Artefacts caused by the bitch's movements give a graph with an intermittent curve; in the worst case, this may hide real uterine contractions. However, it seems that usually it is possible to recognize a contraction despite an intermittent curve. Because a bitch lying on the probe results in a graph pattern similar to a contraction, attention should be paid to the bitch's position. The tocographic probe position in humans is cranially to the navel or slightly laterally to the midline of the abdomen, with an aim to focus on the fundus of the uterus (Euliano et al. 2013). The probe's position in our study was not as accurate but was somewhere in the middle of the horns. The probe's surface is made of self-sticking plastic that prevents the sliding of the probe from the desired position on human skin. This does not work in dogs, probably due to the lack of sweat glands in dog skin. The paper speed affects the graph's pattern, which may influence the interpretation. However, we did not confront interpreting difficulties associated with the paper speed. The slower paper speed (1 cm/min) saves paper and seems to be enough to detect the contractions in the bitch.

6.6 Further studies

Further studies should concentrate on clarifying why the myometrium is not contracting in CUI despite the OXTR upregulation. One reason may simply be a mechanical disability in the case of overstretching. The other reason could be too little stretch of the uterus due to small litter size. Study II had only two large and two small litters in the CUI group; no further conclusions can be drawn about overstretching or too little stretching with current data. It would be of interest to compare CUI bitches with large and small litters. Myometrial strips of longitudinal and circular layers could be used to clarify capability of overstretched myometrium to respond to OXT

treatment, because in *in vitro* studies, oxytocin-induced contractions were more evident in the longitudinal than the circular fibres in the myometrium of non-pregnant bitches (Gogny et al. 2010). It is necessary to separate the longitudinal and circular muscular layers to compare the expression of OXTR. There was a different distribution of the OXTR depending on the layer of the muscle and the site in the porcine uterus in the binding studies by Kitazawa et al. (2001). Prolonged influence of OXT may prevent the contractions by desensitization of receptors (Phaneuf et al. 1997) and should be further clarified in cases like PUI, OD, and MD. However, prolongation of parturition may complicate the comparison of different dystocia groups, because the duration of uterine contractions may be challenging to ascertain. Individual differences may exist regarding how fast the uterus fatigues and when the desensitization occurs.

There seems to be a breed predisposition to uterine inertia, for example, in Border Collies, Boxers, Labrador Retrievers, and Shetland Sheepdogs (Darvelid and Linde-Forsberg 1994; Gill 2002; Linde-Forsberg and Persson 2007; Münnich and Kuchenmeister 2009). Therefore, genetic studies should be conducted to clarify the hereditary background in the breeds and lines known to be prone to uterine inertia, and to determine whether it is possible to develop a genetic test for breeding purposes.

The influence of E2 to OXTR expression should be investigated. It is not a common practice in Finland to perform OHE in healthy bitches during oestrus due to the increased risk of haemorrhage, so we did not have samples from oestrous bitches in our study. Uterine samples from bitches during either naturally occurring or induced oestrus should be taken to be able to investigate OXTR expression under the influence of E2. The blood samples from pregnant bitches for E2 analysis should be taken before the prepartum drop occurs, that is 3 or 4 days before parturition. This complicates the tissue sampling, because if CS is performed at that time to obtain uterine samples, the puppy survival may be compromised. However, planned prepartum CS can be performed safely, at least in English Bulldogs and Boerboels, on the 57th day after the beginning of cytological dioestrus (De Cramer KGM and Nöthling JO 2019, 2020). The pregnancy duration is approximately 60 days from ovulation at this point (Holst and Phemister 1974). Prepartum or elective CS should not be considered as a routine practice but as an individual solution when necessary. The emphasis should be in selecting sound individuals that can give birth naturally. Also, the influence of the other pregnancy and parturition related hormones, like relaxin and prolactin, on OXTR expression should be investigated.

Uterine samples obtained from prepartum and peripartum bitches were not histologically evaluated in our study. Degenerative changes of the endometrium are common in bitches with fertility problems (Mir et al. 2013). It should be investigated whether the degenerative or inflammatory changes affect uterine contractility.

The intrauterine pressure catheter is considered the most reliable method in uterine contraction monitoring (Gibb and Arulkumaran 1987). A study with simultaneous external and

intrauterine monitoring, in both horns, with actual pressure measurement should be conducted to gain more information on contraction patterns in the canine uterus. A more recent, non-invasive external method is electrohysterography (EHG), which records the electrical activity of the uterine muscle using surface electrodes applied to the maternal abdomen around the navel (Leman et al. 1999; Euliano et al. 2013). EHG correlates well with the monitoring of intrauterine pressure and has also been proven to be more reliable than CTG in obese patients (Euliano et al. 2013). In mares, uterine contractions have been investigated by using transrectal ultrasound with B-mode (Sinnemaa et al. 2005) and M-mode (Campbell and England 2004). It probably is possible to detect canine uterine contractions with ultrasonography, but CTG gives more freedom to the bitch to move, and a longer observation period is possible.

A foetal scalp electrode is used in human medicine when external monitoring of foetal heartbeats is disturbed (Ayres-de-Campos et al. 2015). To our knowledge, this method has not been used in dogs, and it may be challenging due to the small size of the bitch, length of the vagina, and the number of foetuses. It remains to be clarified what kind of frequency of contractions is required for the normal progress of parturition in bitches. Uterine contractility in specific clinical cases such as uterine torsion or rupture would also be of interest. However, no typical pattern in tocography has been repeatedly reported for uterine rupture in human medicine (Vlemminx et al. 2017).

7 Conclusions

The study's conclusions:

- Despite the apparent inactivity, the uterus of the non-pregnant bitch expresses oxytocin receptors, but the relative expression is lower than in the pregnant bitches.
- Oxytocin might contribute to endometrial glandular epithelium activity in non-pregnant bitches.
- Endometrial luminal epithelium seems to have more oxytocin-dependent activity during dioestrus than in anoestrus.
- Progesterone does not seem to affect oxytocin receptor expression in the uterus of non-pregnant bitches.
- The gradual decrease of progesterone in late pregnancy is more likely involved in oxytocin receptor upregulation than sudden parturition drop, because the increase in relative expression of oxytocin receptors seems to occur before the sudden parturition decline of progesterone.
- Oxytocin receptors seem to be more abundant in myometrium in pregnant than in non-pregnant bitches.
- A decrease in oxytocin receptors observed in obstructive dystocia but not in complete primary uterine inertia may indicate a different aetiology in a cause of dystocia.
- Human cardiotocography can be used to assist in diagnosing uterine inertia and monitoring the response to uterotonic medicines.

References

- Aguilar HN, Mitchell BF. Physiological pathways and molecular mechanisms regulating uterine contractility. *Hum Reprod Update*. 2010;16:725-44.
- Alexander DP, Britton HG, James VH, Nixon DA, Parker RA, Wintour EM, Wright RD. Steroid secretion by the adrenal gland of foetal and neonatal sheep. *The Journal of Endocrinology*. 1968; 40:1-13.
- Altura BM, Altura BT. Actions of vasopressin, oxytocin, and synthetic analogs on vascular smooth muscle. *Fed Proc*. 1984;43:80-6.
- Arrowsmith S, Wray S. Oxytocin: Its mechanism of action and receptor signalling in the myometrium. *J Neuroendocrinol*. 2014;26:356-69.
- Assali NS, Dasgupta K, Kolin A, Holms L. Measurement of uterine blood flow and uterine metabolism. V. Changes during spontaneous and induced labor in unanesthetized pregnant sheep and dogs. *Am J Physiol*. 1958;195:614-20.
- Ayres-de-Campos D, Spong CY, Chandrachan E, FIGO intrapartum fetal monitoring expert consensus panel. FIGO consensus guidelines on intrapartum fetal monitoring: Cardiotocography. *Int J Gynaecol Obstet*. 2015;131:13-24.
- Batra S. Effect of oxytocin on calcium influx and efflux in the rat myometrium. *Eur J Pharmacol*. 1986;120:57-61.
- Beard AP, Lamming GE. Oestradiol concentration and the development of the uterine oxytocin receptor and oxytocin-induced PGF2 alpha release in ewes. *J Reprod Fertil*. 1994;100:469-75.
- Beccaglia M, Luvoni GC. Prediction of parturition in dogs and cats: Accuracy at different gestational ages. *Reprod Domest Anim*. 2012;47 (Suppl 6):194-6.
- Bergström A, Fransson B, Lagerstedt AS, Kindahl H, Olsson U, Olsson K. Hormonal concentrations in bitches with primary uterine inertia. *Theriogenology*. 2010;73: 1068-75.
- Bergström A, Fransson B, Lagerstedt AS, Olsson K. Primary uterine inertia in 27 bitches: Aetiology and treatment. *J Small Anim Pract*. 2006;47:456-60.
- Bergström A, Nødtvedt A, Lagerstedt AS, Egenvall A. Incidence and breed predilection for dystocia and risk factors for Cesarean section in a Swedish population of insured dogs. *Vet Surg*. 2006;35:786-91.
- Bhatti SF, Rao NA, Okkens AC, Mol JA, Duchateau L, Ducatelle R, van den Ingh TS, Tshamala M, Van Ham LM, Coryn M, Rijnberk A, Kooistra HS. Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch. *Domest Anim Endocrinol*. 2007;33:294-312.
- Biyikli NK, Tuğtepe H, Sener G, Velioğlu-Oğünç A, Cetinel S, Midillioğlu S, Gedik N, Yeğen BC. Oxytocin alleviates oxidative renal injury in pyelonephritic rats via a neutrophil-dependent mechanism. *Peptides*. 2006;27:2249-57.
- Bleicher N. Behavior of the bitch during parturition. *J Am Vet Med Assoc*. 1962;140:1076-82.

Bolis B, Prandi A, Rota A, Faustini M, Veronesi MC. Cortisol fetal fluid concentrations in term pregnancy of small-sized purebred dogs and its preliminary relation to first 24 hours survival of newborns. *Theriogenology*. 2017;88:264-9.

Campbell ML, England GC. Effect of teasing, mechanical stimulation and the intrauterine infusion of saline on uterine contractions in mares. *Vet Rec*. 2004;155:103-10.

Christie DW, Bailey JB, Bell ET, 1972: Classification of cell types in vaginal smears during the canine estrus cycle. *Br vet J*. 1972;128:301-10.

Concannon PW, Butler WR, Hansel W, Knight PJ, Hamilton JM. Parturition and lactation in the bitch: Serum progesterone, cortisol and prolactin. *Biol Reprod*. 1978;19:1113-8.

Concannon PW, Hansel W, Visek WJ. The ovarian cycle of the bitch: Plasma estrogen, LH and progesterone. *Biol Reprod*. 1975;13:112-21.

Concannon PW, Isaman L, Frank DA, Michel FJ, Currie WB. Elevated concentrations of 13,14-dihydro-15-keto-prostaglandin F-2 alpha in maternal plasma during prepartum luteolysis and parturition in dogs (*Canis familiaris*). *J Reprod Fertil*. 1988;84:71-7.

Concannon PW, McCann JP, Temple M. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *J Reprod Fertil Suppl*. 1989;39:3-25.

Concannon P, Whaley S, Lein D, Wissler R. Canine gestation length: Variation related to time of mating and fertile life of sperm. *Am J Vet Res*. 1983;44:1819-21.

Cornelius AJ, Moxon R, Russenberger J, Havlena B, Cheong SH. Identifying risk factors for canine dystocia and stillbirths. *Theriogenology*. 2019;128:201-206

Dalrymple A, Mahn K, Poston L, Songu-Mize E, Tribe RM. Mechanical stretch regulates TRPC expression and calcium entry in human myometrial smooth muscle cells. *Mol Hum Reprod*. 2007;13:171-9.

Darvelid AW, Linde-Forsberg C. Dystocia in the bitch: A retrospective study of 182 cases. *J Small Anim Pract*. 1994;35:402-7.

Davidson AP. Uterine and fetal monitoring in the bitch. *Vet Clin North Am Small Anim Pract*. 2001;31:305-13.

Davidson A. Problems during and after parturition. In: England G, von Heimendahl A, editors. *BSAVA Manual of canine and feline reproduction and neonatology*, Gloucester: British Small Animal Veterinary Association. 2010; pp. 121–34.

De Cramer KGM, Nöthling, JO. The precision of predicting the time of onset of parturition in the bitch using the level of progesterone in plasma during the preparturient period. *Theriogenology*. 2018;107:211-8.

De Cramer KGM, Nöthling, JO. Curtailing parturition observation and performing preparturient cesarean section in bitches. *Theriogenology*. 2019;124:57-64

De Cramer KGM, Nöthling, JO. Towards scheduled pre-parturient caesarean sections in bitches. *Reprod Domest Anim*. 2020; doi: 10.1111/rda.13669. Online ahead of print.

Derussi AA, de Souza RW, Volpato R, Guaitolini CR, Ackermann CL, Taffarel MO, Cardoso GS, Dal-Pai-Silva M, Lopes MD. Progesterone (PR), oestrogen (ER-alpha and ER-beta) and oxytocin

(OTR) gene expression in the oviduct and uterus of pregnant and non-pregnant bitches. *Reprod Domest Anim.* 2012;47 (Suppl 6):197-9.

Eiler H, Armstrong-Backus CS, Lyke WA. Desensitization of rabbit myometrium to oxytocin and prostaglandin F2 alpha. *Am J Physiol.* 1989;257(1 Pt 1):E20-6.

Eilts BE, Davidson AP, Hosgood G, Paccamonti DL, Baker DG. Factors affecting gestation duration in the bitch. *Theriogenology.* 2005;64:242-51.

England GC, Burgess CM, Freeman SL, Smith SC, Pacey AA. Relationship between the fertile period and sperm transport in the bitch. *Theriogenology.* 2006;66:410-8.

Engström T, Atke A, Vilhardt H. Oxytocin receptors and contractile response of the myometrium after long term infusion of prostaglandin F2 alpha, indomethacin, oxytocin and an oxytocin antagonist in rats. *Regul Pept.* 1988;20:65-72.

Euliano TY, Nguyen MT, Darmanjian S, McGorray SP, Euliano N, Onkala A, Gregg AR. Monitoring uterine activity during labor: a comparison of 3 methods. *Am J Obstet Gynecol.* 2013;208:66.e1-6.

Evans KM, Adams VJ. Proportion of litters of purebred dogs born by caesarean section. *J Small Anim Pract.* 2010;51:113-8.

Ferguson JKW. A study of the motility of the intact uterus at term. *Surg Gynecol Obstet.* 1941;73:359-66.

Fuchs AR, Periyasamy S, Soloff MS. Systemic and local regulation of oxytocin receptors in the rat uterus, and their functional significance. *Can J Biochem Cell Biol.* 1983;61:615-24.

Fuchs AR, Helmer H, Behrens O, Liu HC, Antonian L, Chang SM, Fields MJ. Oxytocin and bovine parturition: A steep rise in endometrial oxytocin receptors precedes onset of labor. *Biol Reprod.* 1992;47:937-44.

Galabova G, Egerbacher M, Aurich JE, Leitner M, Walter I. Morphological changes of the endometrial epithelium in the bitch during metoestrus and anoestrus. *Reprod Domest Anim.* 2003;38:415-20.

Gaudet DA. Retrospective Study of 128 Cases of canine dystocia. *J Am Anim Hosp Assoc.* 1985;21:813-18.

Gibb DM, Arulkumaran S. Assessment and management of uterine contractions. *Baillière's Clin Obstet Gynaecol.* 1987;1:111-29.

Gill, M. Perinatal and late neonatal mortality in the dog. PhD thesis. University of Sydney. 2002.

Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. *Physiol Rev.* 2001;81:629-83.

Ginther OJ, Silva LA, Araujo RR, Beg MA. Temporal associations among pulses of 13,14-dihydro-15-keto-PGF2alpha, luteal blood flow, and luteolysis in cattle. *Biol Reprod.* 2007;76:506-13.

Gogny A, Mallem Y, Destrumelle S, Thorin C, Desfontis JC, Gogny M, Fiéni F. In vitro comparison of myometrial contractility induced by aglepristone-oxytocin and aglepristone-PGF2alpha combinations at different stages of the estrus cycle in the bitch. *Theriogenology.* 2010;74:1531-8.

Gram A, Boos A, Kowalewski MP. Uterine and placental expression of canine oxytocin receptor during pregnancy and normal and induced parturition. *Reprod Domest Anim.* 2014(a);49 (Suppl 2):41-9.

Gram A, Büchler U, Boos A, Hoffmann B, Kowalewski MP. Biosynthesis and degradation of canine placental prostaglandins: parturition changes in expression and function of prostaglandin F2 α -synthase (PGFS, AKR1C3) and 15-hydroxyprostaglandin dehydrogenase (HPGD). *Biol Reprod.* 2013;89:2.

Gram A, Fox B, Buchler U, Boos A, Hoffmann B, Kowalewski MP. Canine placental prostaglandin E2 synthase: expression, localization, and biological functions in providing substrates for parturition PGF2 α synthesis. *Biol Reprod.* 2014(b);91:154.

Groppetti D, Pecile A, Del Carro AP, Copley K, Minero M, Cremonesi F. Evaluation of newborn canine viability by means of umbilical vein lactate measurement, apgar score and uterine tocodynamometry. *Theriogenology.* 2010;74:1187-96.

Haterius HO, Ferguson JKW. Evidence for the hormonal nature of the oxytocic principle of the hypophysis. *Am J Physiol.* 1938;124:314-21.

Hoffmann B, Busges F, Engel E, Kowalewski MP, Papa PC. Regulation of corpus luteum-function in the bitch. *Reprod Domest Anim.* 2004;39:232–40.

Hoffmann B, Höveler R, Hasan SH, Failing K. Ovarian and pituitary function in dogs after hysterectomy. *J Reprod Fertil.* 1992;96:837-45.

Hoffmann B, Höveler R, Nohr B, Hasan SH. Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens. *Exp Clin Endocrinol.* 1994;102:185-9.

Holst PA, Phemister RD. Onset of diestrus in the beagle bitch: Definition and significance. *Am J Vet Res.* 1974;35:401-6.

Hydbring E, Madej A, MacDonald E, Drugge-Boholm G, Berglund B, Olsson K. Hormonal changes during parturition in heifers and goats are related to the phases and severity of labour. *J Endocrinol.* 1999;160:75-85.

Ibuki R, Haga N, Muramatsu S, Mizumoto A, Itoh Z. Long-term observations of uterine contractions in nonpregnant dogs. *Biol Reprod.* 1997;56:632-9.

Irons PC, Nöthling JO, Volkmann DH. Failure of luteolysis leads to prolonged gestation in a bitch: a case report. *Theriogenology.* 1997;48:353-9.

Işeri SO, Sener G, Sağlam B, Gedik N, Ercan F, Yeğen BC. Oxytocin ameliorates oxidative colonic inflammation by a neutrophil-dependent mechanism. *Peptides.* 2005;26:483-91.

Jackson BT, Piasecki GJ. Fetal secretion of glucocorticoids. *Endocrinology.* 1969;85:875-80.

Jackson BT, Rauschecker HF, Piasecki GJ. Quantitative relations of fetal and maternal pituitary-adrenal systems. *J Clin Invest.* 1973;52:3154-60.

Johnston SD, Root Kustritz MV, Olson PNS. Canine and feline theriogenology, 1st ed. PA Saunders, Philadelphia. 2001; pp. 66-128.

- Katusic ZS, Shepherd JT, Vanhoutte PM. Oxytocin causes endothelium-dependent relaxations of canine basilar arteries by activating V1-vasopressinergic receptors. *J Pharmacol Exp Ther*. 1986;236:166-70.
- Kendrick KM, Keverne EB, Hinton MR, Goode JA. Cerebrospinal fluid and plasma concentrations of oxytocin and vasopressin during parturition and vaginocervical stimulation in the sheep. *Brain Res Bull*. 1991;26:803-7.
- Kitazawa T, Kajiwara T, Kiuchi A, Hatakeyama H, Taneike T. Muscle layer- and region-dependent distributions of oxytocin receptors in the porcine myometrium. *Peptides*. 2001;22:963-74.
- Klarenbeek M, Okkens AC, Kooistra HS, Mol JA, Bevers MM, Taverne MAM. Plasma oxytocin concentrations during late pregnancy and parturition in the dog. *Theriogenology*. 2007;68:1169-76.
- Kowalewski MP. Luteal regression vs. prepartum luteolysis: Regulatory mechanisms governing canine corpus luteum function. *Reprod Biol*. 2014(a);14:89-102.
- Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Küçükaslan I, Hoffmann B. Canine placenta: a source of prepartal prostaglandins during normal and antiprogesterin-induced parturition. *Reproduction*. 2010;139:655-64.
- Kowalewski MP, Gram A, Kautz E, Graubner FR. The dog: Nonconformist, not only in maternal recognition signaling. In: Geisert RD, Bazer FW, editors. *Regulation of implantation and establishment of pregnancy in mammals, advances in anatomy, embryology and cell biology*. Springer, Switzerland. 2015; pp. 215-37.
- Kowalewski MP, Kautz E, Högger E, Hoffmann B, Boos A. Interplacental uterine expression of genes involved in prostaglandin synthesis during canine pregnancy and at induced prepartum luteolysis/abortion. *Reprod Biol Endocrinol*. 2014(b);12:46.
- Leman H, Marque C, Gondry J. Use of the electrohysterogram signal for characterization of contractions during pregnancy. *IEEE Trans Biomed Eng*. 1999;46:1222-9.
- Liggins GC, Fairclough RJ, Grieves SA, Kendall JZ, Knox BS. The mechanism of initiation of parturition in the ewe. *Recent Prog Horm Res*. 1973;29:111-59.
- Liggins GC, Kennedy PC, Holm LW. Failure of initiation of parturition after electrocoagulation of the pituitary of the fetal lamb. *Am J Obstet Gynecol*. 1967;98:1080-6.
- Linde-Forsberg C. Abnormalities in pregnancy, parturition, and the periparturient period. In: Ettinger, SJ, Feldman EC, editors. *Textbook of veterinary internal medicine*, 7th ed., Saunders, St. Louis, MO. 2010; pp. 1890–901.
- Linde Forsberg C, Persson G. A survey of dystocia in the Boxer breed. *Acta Vet Scand*. 2007;49:8.
- Lundin-Schiller S, Kreider DL, Rorie RW, Hardesty D, Mitchell MD, Koike TI. Characterization of porcine endometrial, myometrial, and mammary oxytocin binding sites during gestation and labor. *Biol Reprod*. 1996;55:575-81.
- Luvoni GC, Beccaglia M. The prediction of parturition date in canine pregnancy. *Reprod Domest Anim*. 2006;41:27-32.
- Luz MR, Bertan CM, Binelli M, Lopes MD. Plasma concentrations of 13,14-dihydro-15-keto prostaglandin F2-alpha (PGFM), progesterone and estradiol in pregnant and nonpregnant diestrus cross-bred bitches. *Theriogenology*. 2006;66:1436-41.

Macones GA, Hankins GD, Spong CY, Hauth J, Moore T. The 2008 National Institute of Child Health and Human Development workshop report on electronic fetal monitoring: update on definitions, interpretation, and research guidelines. *Obstet Gynecol.* 2008;112:661-6.

Mariani TC, do Prado C, Silva LG, Paarmann FA, Lima MC, Carvalho I, Campos DB, Artoni LP, Hernandez-Blazquez FJ, Papa PC. Immunohistochemical localization of VEGF and its receptors in the corpus luteum of the bitch during diestrus and anestrus. *Theriogenology.* 2006;66:1715–20.

Miles AM, Monga M, Richeson KS. Correlation of external and internal monitoring of uterine activity in a cohort of term patients. *Am J Perinatol.* 2001;18:137-40.

Mir F, Fontaine E, Albaric O, Greer M, Vannier F, Schlafer DH, Fontbonne A. Findings in uterine biopsies obtained by laparotomy from bitches with unexplained infertility or pregnancy loss: an observational study. *Theriogenology.* 2013;79:312-22.

Münnich A, Küchenmeister U. Dystocia in Numbers – Evidence-based parameters for intervention in the dog: causes for dystocia and treatment recommendations. *Reprod Domest Anim.* 2009;44 (Suppl. 2):141–7.

Ndiaye K, Poole DH, Pate JL. Expression and regulation of functional oxytocin receptors in bovine T lymphocytes. *Biol Reprod.* 2008;78:786-93.

Nickel R, Sack WO, Schummer E, Seiferle E. The viscera of the domestic mammals. 2nd rev ed, Parey, Berlin, 1979; pp. 351-92.

Nohr B, Hoffmann B, Steinetz BE. Investigation of the endocrine control of parturition in the dog by application of an antigestagen. *J Reprod Fertil Suppl.* 1993;47:542–3.

Nurani, R., Chandrarahan, E., Lowe, V., Ugwumadu, A., & Arulkumaran, S. Misidentification of maternal heart rate as fetal on cardiotocography during the second stage of labour: the role of the fetal electrocardiograph. *Acta Obstet Gynecol Scand.* 2012;91:1428-32.

Okkens AC, Dieleman SJ, Bevers MM, Lubberink AA, Willemse AH. Influence of hypophysectomy on the lifespan of the corpus luteum in the cyclic dog. *J Reprod Fertil.* 1986;77:187–92.

Olsson K, Bergström A, Kindahl H, Lagerstedt A-S. Increased plasma concentrations of vasopressin, oxytocin, cortisol and the prostaglandin F2 α metabolite during labour in the dog. *Acta Physiol Scand.* 2003;179:281-7.

Onclin K, Murphy B, Verstegen JP. Comparisons of estradiol, LH and FSH patterns in pregnant and nonpregnant beagle bitches. *Theriogenology.* 2002;57:1957-72.

O'Neill DG, O'sullivan AM, Manson EA, Church DB, Boag AK, McGreevy PD, Brodbelt DC. Canine dystocia in 50 UK first-opinion emergency care veterinary practices: prevalence and risk factors. *Vet Rec.* 2017;181:88.

Ou CW, Chen ZQ, Qi S, Lye SJ. Increased expression of the rat myometrial oxytocin receptor messenger ribonucleic acid during labor requires both mechanical and hormonal signals. *Biol Reprod.* 1998;59:1055-61.

Petersson M, Wiberg U, Lundeberg T, Uvnäs-Moberg K. Oxytocin decreases carrageenan induced inflammation in rats. *Peptides.* 2001;22:1479-84.

Phaneuf S, Asbóth G, Carrasco MP, Europe-Finner GN, Saji F, Kimura T, Harris A, López Bernal A. The desensitization of oxytocin receptors in human myometrial cells is accompanied by down-regulation of oxytocin receptor messenger RNA. *J Endocrinol.* 1997;154:7-18.

Popescu LM, Nutu O, Panoiu C. Oxytocin contracts the human uterus at term by inhibiting the myometrial Ca²⁺-extrusion pump. *Biosci Rep.* 1985;5:21-8.

Ragusa A, de Carolis C, dal Lago A, Miriello D, Ruggiero G, Brucato A, Pisoni MP, Muscarà M, Merati R, Maccario L, Nobili M. Progesterone supplement in pregnancy: An immunologic therapy? *Lupus.* 2004;13:639-42.

Rehm S, Stanislaus DJ, Williams AM. Estrous cycle-dependent histology and review of sex steroid receptor expression in dog reproductive tissues and mammary gland and associated hormone levels. *Birth Defects Res B Dev Reprod Toxicol.* 2007;80:233-45.

Richard S, Zingg HH. The human oxytocin gene promoter is regulated by estrogens. *J Biol Chem.* 1990;265:6098-103.

Robertson HA. Changes in the concentration of unconjugated oestrone, oestradiol-17 α and oestradiol-17 β in the maternal plasma of the pregnant cow in relation to the initiation of parturition and lactation. *J Reprod Fertil.* 1974;36:1-7.

Robertson HA, Smeaton TC. The concentration of unconjugated oestrone, oestradiol-17 α and oestradiol-17 β in the maternal plasma of the pregnant ewe in relation to the initiation of parturition and lactation. *J Reprod Fertil.* 1973;35:461-8.

Robinson RS, Mann GE, Lamming GE, Wathes DC. The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. *J Endocrinol.* 1999;160:21-33.

Robinson RS, Mann GE, Lamming GE, Wathes DC. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. *Reproduction.* 2001;122:965-79.

Sato M, Noguchi J, Mashima M, Tanaka H, Hata T. 3D power Doppler ultrasound assessment of placental perfusion during uterine contraction in labor. *Placenta.* 2016;45:32-6.

Schröder M, Münnich A, Falkenberg U, Heuwieser W. Tocodynamometry as a non-invasive method for monitoring labour patterns, delivery and dystocia in the dog to reduce neonatal mortality. *Reprod Domest Anim.* 2006;41 (Suppl. 1):31.

Silva E, Leitão S, Ferreira-Dias G, Lopes da Costa L, Mateus L. Prostaglandin synthesis genes are differentially transcribed in normal and pyometra endometria of bitches. *Reprod Domest Anim.* 2009;44 (Suppl 2):200-3.

Sinnemaa L, Järvinen T, Lehmonen N, Mäkelä O, Reilas T, Sankari S, Katila T. (2005). Effect of insemination volume on uterine contractions and inflammatory response and on elimination of semen in the mare uterus. *J Vet Med A Physiol Pathol Clin Med.* 2005;52:466–71.

Sokolowski JH. The effects of ovariectomy on pregnancy maintenance in the bitch. *Lab Anim Sci.* 1971;21:696-9.

Starbuck GR, Stout TA, Lamming GE, Allen WR, Flint AP. Endometrial oxytocin receptor and uterine prostaglandin secretion in mares during the oestrous cycle and early pregnancy. *J Reprod Fertil.* 1998;113:173-9.

Taverne M, Noakes DE. Parturition and the care of parturient animals and the newborn. In: Noakes DE, Parkinson T, England G, editors. *Veterinary Reproduction and Obstetrics*, 10th ed. Elsevier Ltd. 2019; pp. 115-47.

Tsutsui T. Effects of ovariectomy and progesterone treatment on the maintenance of pregnancy in bitches. *Nihon Juigaku Zasshi*. 1983;45:47-51.

Tsutsui T, Kawakami E, Murao I, Ogasa A. Transport of spermatozoa in the reproductive tract of the bitch: observations through uterine fistula. *Nihon Juigaku Zasshi*. 1989;51:560-5.

Tsutsui T, Stewart DR. Determination of the source of relaxin immunoreactivity during pregnancy in the dog. *J Vet Med Sci*. 1991;53:1025-9.

Tønnessen R, Sverdrup Borge K, Nødtvedt A, Indrebø A. Canine perinatal mortality: A cohort study of 224 breeds. *Theriogenology*. 2012;77:1788-1801.

van der Weyden GC, Taverne MA, Dieleman SJ, Wurth Y, Bevers MM, van Oord HA. Physiological aspects of pregnancy and parturition in dogs. *J Reprod Fertil Suppl*. 1989;39:211-24.

Vannuccini S, Bocchi C, Severi FM, Challis JR, Petraglia F. Endocrinology of human parturition. *Ann Endocrinol (Paris)*. 2016;77:105-13.

VectorStock website of oxytocin molecule: <https://www.vectorstock.com/royalty-free-vector/chemical-formula-of-the-oxytocin-molecule-vector-21864137>. Fetched on 9.12.2019.

Veiga GA, Milazzotto MP, Nichi M, Lúcio CF, Silva LC, Angrimani DS, Vannucchi CI. Gene expression of estrogen and oxytocin receptors in the uterus of pregnant and parturient bitches. *Braz J Med Biol Res*. 2015;48:339-43.

Veronesi MC, Battocchio M, Marinelli L, Faustini M, Kindahl H, Cairolì F. Correlations among body temperature, plasma progesterone, cortisol and prostaglandin F2 α of the periparturient bitch. *J Vet Med A*. 2002;49:264-8.

Vlemminx MW, de Lau H, Oei SG. Tocogram characteristics of uterine rupture: a systematic review. *Arch Gynecol Obstet*. 2017;295:17-26.

Wathes DC, Smith HF, Leung ST, Stevenson KR, Meier S, Jenkin G. Oxytocin receptor development in ovine uterus and cervix throughout pregnancy and at parturition as determined by in situ hybridization analysis. *J Reprod Fertil*. 1996;106:23-31.

Weeks AD. The retained placenta. *Best Pract Res Clin Obstet Gynaecol*. 2008;22:1103-17.

Wheaton LG, Pijanowski GJ, Weston PG, Burke TJ. Uterine motility during the estrous cycle: studies in healthy bitches. *Am J Vet Res*. 1988;49:82-6.

Wu W, Nathanielsz PW. Changes in oxytocin receptor messenger RNA in the endometrium, myometrium, mesometrium, and cervix of sheep in late gestation and during spontaneous and cortisol-induced labor. *J Soc Gynecol Investig*. 1994;1:191-6.

Wu WX, Verbalis JG, Hoffman GE, Derks JB, Nathanielsz PW. Characterization of oxytocin receptor expression and distribution in the pregnant sheep uterus. *Endocrinology*. 1996;137:722-8.

Yoshinaga K, Hawkins RA, Stocker JF. Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. *Endocrinology*. 1969;85:103-12.

Zingg HH, Rozen F, Chu K, Larcher A, Arslan A, Richard S, Lefèbvre D. Oxytocin and oxytocin receptor gene expression in the uterus. *Recent Prog Horm Res.* 1995;50:255-73.

Zingg HH, Laporte SA. The oxytocin receptor. *Trends Endocrinol Metab.* 2003;14:222-7.

Original articles

I

Oxytocin receptors in dioestrous and anoestrous canine uteri

TM Tamminen¹ | L Sahlin² | B Masironi² | J Taponen¹ | O Laitinen-Vapaavuori³ | T Katila¹

¹Department of Production Animal Medicine, University of Helsinki, Saarentaus, Finland

²Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

³Department of Equine and Small Animal Medicine, University of Helsinki, Helsinki, Finland

Correspondence

Tuire M. Tamminen, Department of Production Animal Medicine, University of Helsinki, Saarentaus, Finland.
Email: tuire.tamminen@helsinki.fi

Funding information

Finnish Foundation of Veterinary Research; Finnish Veterinary Foundation; European Veterinary Society for Small Animal Reproduction (EVSSAR)

Contents

The aim of the study was to localize oxytocin receptors (OTR) and measure mRNA expression of OTR in the canine uterus with and without the influence of progesterone. Uterine samples were taken from nine anoestrous and eight dioestrous bitches during ovariectomy. Histological changes were evaluated in haematoxylin and eosin (HE)-stained samples. Purified polyclonal antibody for OTR was used in immunohistochemistry to localize receptors in uterine layers. Relative mRNA concentration of OTR was evaluated with real-time PCR from full-thickness uterine samples taken from the middle horn and the body. Myometrial smooth muscle cells, endometrial luminal epithelium (LE) and deep and superficial glandular epithelium were positively stained for oxytocin receptors in non-pregnant animals. No significant difference in staining intensity was detected between uterine middle horn and body. However, the staining intensity of LE was significantly higher in dioestrous than in anoestrous uteri ($p < .05$). Leucocytes and endothelium of blood vessels were also positively stained for OTR. Real-time PCR showed no significant differences in OTR mRNA expression between the middle horn and the body of the uterus, or between anoestrous and dioestrous uterus. No correlation was noted between OTR mRNA expression and blood progesterone concentration. In conclusion, despite the apparent inactivity, the uterus of the non-pregnant bitch expresses OTR. The distribution or relative expression of OTR does not differ between uterine horn and body in dioestrus or anoestrus except in LE. LE may have more oxytocin-dependent activity during dioestrus than anoestrus.

1 | INTRODUCTION

Oxytocin (OT), a nonapeptide hormone, is released from the posterior pituitary gland into systemic circulation following a suitable stimulus, such as cervical pressure during parturition. The effect of OT in the target tissue is mediated by specific G-protein-coupled transmembrane receptors, oxytocin receptors (OTR). In the uterus, the binding of OT to its receptors leads to uterine contractions that are essential for parturition to proceed. In addition to uterine and mammary tissue, OT and OTR are found for example in the central nervous system, testis, prostate gland, kidney and heart (Gimpl & Fahrenholz, 2001). Uterine contractions and milk let-down are the most familiar effects of OT, but it has a diverse role in other tissues and also in behaviour (Gimpl & Fahrenholz, 2001).

Recent studies of OTR in canine uterus have shown that the expression of OTR mRNA is higher in the uterus of pregnant bitches near term compared with earlier stages of pregnancy, which indicates that OTR are upregulated at the time of parturition luteolysis (Derussi et al., 2012; Gram, Boos, & Kowalewski, 2014). Antigestagen treatment in mid-pregnancy increased expression of OTR mRNA in the uterus of pregnant bitches (Gram et al., 2014). These studies indicate that progesterone has a regulatory role in OTR expression in dogs. Gene expression of OT and OTR is, in many species, regulated by oestrogens and progesterone (Beard & Lamming, 1994; Richard & Zingg, 1990; Spencer, Johnson, Burghardt, & Bazer, 2004).

In most non-pregnant mammals, uterine secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is necessary for luteolysis (Niswender, Juengel, Silva, Rollyson, & McIntush, 2000). Corpora lutea (CL) secreting

progesterone, $\text{PGF}_{2\alpha}$ and OT (Skarzynski & Okuda, 1999) and expression of endometrial OTR (Robinson, Mann, Lamming, & Wathes, 2001) are also involved in the regulation of luteolysis in cows. However, in non-pregnant bitches, luteal prostaglandins are probably more involved in the development than in the regression of CL (Kowalewski, 2014) and the luteal regression occurs due to CL ageing without uterine luteolysin (Hoffmann, Höveler, Hasan, & Failing, 1992).

In OT binding studies, OTR were present in the myometrium of non-pregnant gilts, and the distribution of the OTR differed depending on the site of the uterus and the layer of the muscle (Kitazawa, Kajiwara, Kiuchi, Hatakeyama, & Taneike, 2001). Besides myometrium, the presence of OTR has been reported in the endometrium of cows (Robinson, Mann, Lamming, & Wathes, 1999; Robinson et al., 2001), ewes (Wu, Verbalis, Hoffman, Derks, & Nathanielsz, 1996) and mares (Starbuck, Stout, Lamming, Allen, & Flint, 1998).

Basic research is needed in the regulation of OT and OTR systems in non-pregnant and pregnant bitches to understand the mechanisms behind uterine contractility. The uterus is under the influence of progesterone during dioestrus, whereas during anoestrus, the progesterone is at the basal level, thus creating an entirely different regulatory environment. The aim of this study was to localize and semiquantitatively evaluate, using immunohistochemistry (IHC), the existence of OTR in canine uterine tissue during dioestrus and anoestrus. Real-time PCR was performed to estimate the relative mRNA concentrations of OTR. In addition, the histological changes were evaluated.

2 | MATERIALS AND METHODS

2.1 | Animals

A total of 17 client-owned pet bitches of different breeds were included in the study (Table 1). Bitches were brought to the Veterinary Teaching Hospital of the University of Helsinki, Saari Unit, for ovariectomy. Inclusion criteria were ovariectomy without signs of pyometra and an oral consent from the owner to use the removed tissue. The study was approved by the Ethical Committee of the Viikki Campus, University of Helsinki.

2.2 | Stage of the oestrous cycle

The stage of the oestrous cycle was determined by the history of the last oestrus, inspection of vulvar oedema and discharge, vaginal cytology, serum progesterone concentration and macroscopic findings of ovaries according to Christie, Bailey, and Bell (1972) and Concannon, Hansel, and Visek (1975). A vaginal smear consisting mainly of parabasal and intermediate epithelial cells, progesterone concentration below 6 nmol/L, no vulvar oedema or discharge and no follicles present in ovaries indicated anoestrus. A vaginal smear containing <30% of cornified superficial cells, progesterone concentration over 6 nmol/L, no vulvar oedema or discharge and no follicles but corpora lutea (CL) present in ovaries were indicative of dioestrus. In one case, the vaginal smear and progesterone analysis were not performed. That bitch had been mated once during a visibly normal heat 19 days before

ovariohysterectomy. Embryos were not evident when the uterine lavage fluid was evaluated using light microscopy nor when the uterus was inspected after incision. Several CL were seen in the ovaries. The bitch was included in the dioestrous group.

2.3 | Blood sampling and progesterone measurements

Blood samples were taken prior to surgery from the vena cephalica into serum tubes with clotting activator (Vacuette®, Mekalasi Oy, Nurmijärvi, Finland). Serum was frozen at -20°C and stored at -70°C until analysed. Serum progesterone concentrations were determined using a commercial RIA kit (Progesterone Coat-A-Count® RIA, Siemens Healthcare Diagnostics Oy, Espoo, Finland) according to the manufacturer's instructions. The concentrations were determined with a gamma counter (1272 GliniGamma, LKB Wallac Oy, Turku, Finland) as duplicates in one run. The intra-assay coefficient of variation was 3.4% at the level of 4.4 nmol/L and 2.0% at the level of 32.5 nmol/L. The detection limit was 0.3 nmol/L.

2.4 | Uterine samples

Immediately after the removal of the ovaries and uterus, a full-thickness sample was taken from the uterine body or bifurcation (body) and from the middle part of the horn (horn). The sample was divided into two parts: one part was immediately frozen in liquid nitrogen and stored at -70°C for PCR analysis, and the other part was fixed in 4% phosphate-buffered formaldehyde (pH 7.0) for a minimum of 20 hr and maximum of 24 hr and subsequently in 70% ethanol at $+4^{\circ}\text{C}$ until embedded in paraffin, cut into 5- μm sections and mounted on slides (SuperFrost Plus, Thermo Scientific, Gerhard Menzel GmbH, Braunschweig, Germany) for IHC and histological evaluation. In one case, only a horn sample was taken. For PCR, 16 samples were available from both uterine body and horn.

2.5 | Histology

Uterine sections on slides were stained with haematoxylin-eosin (HE) for histological evaluation. Endometrial cysts were recorded. The number of leucocytes was estimated in vessels in myometrium and endometrium as being negative (–), occasional in a few fields (+), occasional in several fields (++) and several in all fields (+++).

2.6 | Immunohistochemistry

Uterine sections on slides were deparaffinized in xylene, rehydrated in a graded ethanol series and rinsed with distilled water and TBS. The sections were boiled in sodium citrate (0.01 M, pH 6.0) in a microwave oven for 10 min. After cooling for 20 min in room temperature and rinsing with TBS, the sections were covered with 3% H_2O_2 in methanol to block endogenous peroxidase. After rinsing with TBS, a commercial blocking reagent (Background Sniper, Biocare Medical LLC, Concord, CA 949520, USA) was used to prevent non-specific

TABLE 1 The individual and average data and results for the 17 bitches divided into anoestrous and dioestrous groups. Macroscopical ovarian (CL: corpora lutea, CLr: regressed corpora lutea) and histological uterine (endometrial cysts, leucocytes in endometrium and myometrium) findings are presented. The immunohistochemical (IHC) staining for oxytocin receptors (OTR) is scored in uterine horn (H) and body (B) in myometrium (MYO), endometrial luminal epithelium (LE) and endometrial glandular epithelium (GE). P₄: progesterone, n/a: not available

Breed	age (years)	weight (kg)	P ₄ nmol/L	CL or CLr	Stage of oestrous cycle	Comments	Leucocytes: endo/myo	Endometrial cysts	IHC scoring for OTR in uterine horn (H) and body (B)					
									H MYO	H LE	H GE	B MYO	B LE	B GE
Airedalen Terrier	1.7, 24.6		0.4	-	Anoestrus	Uterine hyperaemia	+/+		0	0	2	n/a	n/a	n/a
Alaskan Husky	6.2, 23.7		0.4	CLr	Anoestrus	Uterine hyperaemia	++/++	+	2	3	3	0	1	2
Chihuahua	5.4, 2.9		1.4	CLr	Anoestrus	Uterine hyperaemia	+++ /+++	+	1	3	3	0	1	1
Hovawart	7.3, 33.4		1.0	CLr	Anoestrus	Mammary tumours	+/+		2	2	3	2	2	3
Mixed	1.0, 7.7		1.1	-	Anoestrus		+/+		3	1	3	0	1	2
Mixed	2.1, 24.6		0.9	CLr	Anoestrus		+/+		3	1	3	2	1	3
Mixed	2.4, 36.4		0.3	-	Anoestrus		+/+		2	1	3	0	0	2
Mixed	4.2, 18.8		1.0	CLr	Anoestrus		+/+		1	1	3	2	1	3
Mixed	6.3, 17.0		0.4	-	Anoestrus		+/+		3	0	2	3	1	3
Anoestrus average: 4.6 years, 20.9 kg			0.8						1.9	1.3	2.8	1.1	1.0	2.4
Australian Terrier	2.4, 7.2		72.6	CL	Dioestrus		+/+		2	0	2	3	2	3
Finnish Lapphund	3.4, 17.6		n/a	CL	Dioestrus	Mismatched, non-gravid	+/+		2	3	3	3	3	3
Finnish Spitz	4.8, 11.7		6.1	CL	Dioestrus		+/+	+	0	2	1	2	2	2
Mixed	0.8, 22.5		128.0	CL	Dioestrus		+/+		3	3	3	0	2	2
Mixed	0.9, 39.0		8.2	CL	Dioestrus		+++ /+++		0	2	2	2	2	3
Norwich Terrier	8.5, 6.3		27.3	CL	Dioestrus	Uterine fibrosis	+/+		1	2	2	2	1	3
Portuguese Podengo	5.5, 5.1		163.3	CL	Dioestrus	Mismatched, non-gravid	+/+		2	3	3	2	2	2
Welsh Corgi Pembroke	2.2, 14.0		106.4	CL	Dioestrus		++/++		1	2	2	2	3	3
Dioestrus average: 3.6 years, 15.4 kg			73.1						1.4	2.1	2.3	2.0	2.1	2.6

binding. As primary antibody, a purified polyclonal rabbit-anti-human antibody to OTR (ABIN302438, Antibodies Online, Aachen, Germany) was used in 1:200 dilution with TBS. For negative controls, the primary antibody was replaced by an equivalent amount of rabbit IgG. After incubation for one hour at room temperature and subsequent rinsing with TBS and 0.01% Tween20, a biotinylated horse-anti-rabbit antibody was added to the sections as a secondary antibody in 1:500 dilution with 5% normal horse serum in TBS containing 5% BSA, and incubated in the dark for 30 min. After rinsing with TBS and 0.01% Tween20, avidin-biotin complex (Vectastain® Elite ABC Kit, PK6100, Vector Laboratories Ltd, Peterborough, UK) was added and the sections were incubated in the dark for 30 min to enhance signals. After rinsing with TBS and 0.01% Tween20, DAB chromogen substrate (DAKO K3466) was added to achieve brown staining. After rinsing with distilled water, haematoxylin was added for counterstaining. The sections were rinsed with running tap water and dehydrated in a graded ethanol series and xylene.

Immunostaining of OTR was assessed by manual scoring in myometrial (longitudinal and circular layers) smooth muscle cells (MYO), endometrial luminal epithelial cells (LE) and endometrial superficial and deep glandular epithelial cells (GE). The blind scoring was performed by two observers on a four-point scale from negative (0), (+) faint and (++) moderate to (+++) strong immunostaining. The results using this method, and two independent observers, show a good level of consistency between investigators (Isaksson et al., 2003).

2.7 | PCR

2.7.1 | RNA preparation and reverse transcription

The canine uterine samples were relocated from -70 to -20°C and after 24 hr transferred to RNeasy®ICE (Ambion Inc.) and stored at -20°C until cut into 25–30 mg pieces and homogenized. Total RNA from the uterine homogenate was isolated and purified using the RNeasy® Mini kit (Qiagen GmbH, Hilden, Germany) according to the procedure for RNA isolation from fibrous tissues, including a DNase step, as recommended by the manufacturer. Concentration and purity of RNA were determined spectrophotometrically at 260 and 280 nm. The RNA quality was assessed by visualization of 28S and 18S rRNA bands after electrophoresis. Two micrograms of total RNA from each sample was reverse-transcribed at 37°C for 60 min in a final volume of 20 µl with a reaction mixture (Qiagen) containing 1× RT buffer, dNTP mix (0.5 mM each dNTP), 600 ng random primers (Invitrogen, Paisley, UK), two units of RNase inhibitor (Qiagen) and four units of Omniscript™ reverse transcriptase (Qiagen).

2.8 | Real-time PCR analysis

The oligonucleotide primer pair for the OTR was designed with NCBI/Primer-BLAST. To standardize the quantification method, RPL27 and HPRT1 were selected as non-regulated reference genes and the primer pairs were obtained from Silva, Leitão, Ferreira-Dias, Lopes da Costa, and Mateus (2009) and Bhatti et al. (2007), respectively. The primers were

based on the sequences of the canine genes. The primers used were the following: OTR forward primer: 5'-TGCTGGCCTTCATCGTGTGCT-3'; OTR reverse primer: 5'-GATGAAAGCCGAGGCTTCCTTGGG-3' from NM_001198659.1 with predicted size 95 bp; RPL27 forward primer: 5'-ACAATCACCTCATGCCACA-3'; RPL27 reverse primer: 5'-CTTGACCTTGGCCTCTCGTC-3' from NM_001003102.2 with predicted size 122 bp; HPRT1 forward primer: 5'-AGCTTGCTGGTGA AAAGGAC-3'; HPRT1 reverse primer: 5'-TTATAGTCAAGGGC ATATCC-3' from NM_001003357.1 with predicted size 104 bp.

Real-time PCR was performed in an ICYCLER™ IQ real-time PCR System (Bio-Rad Laboratories, Inc). For PCR, the cDNAs corresponding to 50 ng (HPRT1) or 100 ng (OTR and RPL27) RNA were added to 12.5 µl of iQ™ SYBR® Green Supermix (Bio-Rad) and 0.3 µM of each oligonucleotide primer in a final volume of 25 µl. After an initial incubation for 3 min at 95°C, the samples were subjected to 40 cycles of 10 seconds (s) at 95°C, followed by 45 s at 57°C. All samples were run in duplicate. The purity of PCR products was confirmed by a melting curve analysis in all experiments. Each PCR assay included a negative control containing an RNA sample without reverse transcription. The PCR amplification rate and the cycle threshold (Ct) values were analysed using ICYCLER™ IQ 3.1 software (Bio-Rad). The OTR product was normalized against the mean of RPL27 and HPRT1 products to give the relative expression of OTR mRNA.

2.9 | Statistical analysis

The data were analysed using IBM SPSS STATISTICS 23 software for WINDOWS. The nonparametric related-samples Wilcoxon signed-rank test was used for the staining intensity in IHC and relative expression of OTR mRNA between the horn and the body of the uterus, and the nonparametric Mann-Whitney *U*-test, between dioestrous and anoestrous uteri. The differences were considered statistically significant at $p < .05$. The correlation of serum progesterone and expression of OTR mRNA was evaluated with Kendall's Tau.

3 | RESULTS

3.1 | Histology

All layers of uterine tissue were present in all 17 horn and 16 body samples. Vacuolated cytoplasm, due to lipid accumulation, was noticed in luminal epithelium and crypts of the endometrium in six samples (four dioestrous, two anoestrous). Uterine hyperaemia, cysts and fibrotic changes were found in a few cases (Table 1). Fewer crypts and smaller glands were noted in anoestrous than in dioestrous endometrial samples.

3.2 | Immunohistochemistry

The staining for OTR in IHC was observed in the cytoplasm of cells in MYO, LE and GE (Figure 1, Table 1). A weak staining was also noticed in the endometrial stroma. No significant difference in staining intensity in MYO, LE or GE was detected between the horn and the body.

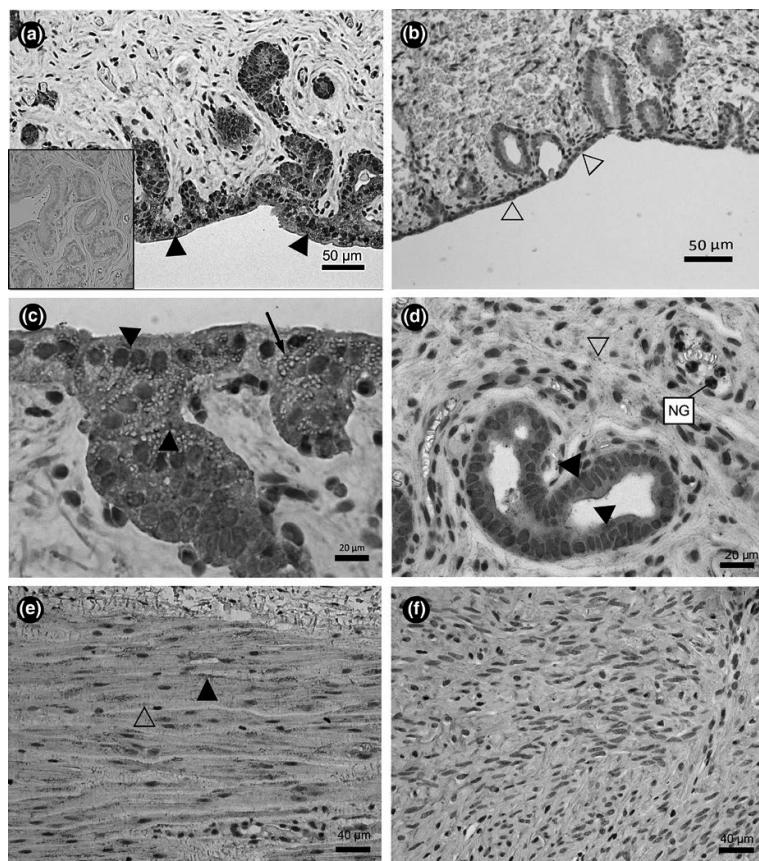


FIGURE 1 Immunohistochemical staining for oxytocin receptor (OTR) in the non-pregnant canine uterus. Brown colour indicates positive staining for OTR. (a) Dioestrus, middle horn, endometrial luminal epithelium: strong signal (solid arrow head). Insert: negative control, no background staining. (b) Anoestrus, middle horn, endometrial luminal epithelium: very weak signal (open arrow head). (c) Dioestrus, middle horn, degenerating endometrial luminal epithelium: strong signal (solid arrow head), vacuoles (arrow). (d) Dioestrus, body, endometrium, glandular epithelium of superficial gland: strong signal (solid arrow head) and stroma: weak signal (open arrow head), neutrophil granulocyte (NG): strong signal. (e) Dioestrus, body, myometrium, circular layer: strong (solid arrow head) and weak (open arrow head) signals. (f) Dioestrus, body, myometrium, longitudinal layer, negative control: no background staining

A significant difference was recorded in the LE between anoestrous and dioestrous uteri: the staining intensity was higher in dioestrous than in anoestrous uteri ($p < .05$). No significant difference was noted in MYO or GE between anoestrous and dioestrous uteri, or between circular and longitudinal layers of MYO. In all horn and body samples, leucocytes and endothelium of blood vessels were positively stained, with the exception of one body sample for which no leucocytes were detected (Figure 1).

3.3 | qPCR

In real-time PCR, no significant difference was detected in OTR mRNA expression between the horn and the body, or between anoestrous and dioestrous uteri (Figure 2). There was no correlation between OTR mRNA expression and the blood progesterone concentration.

4 | DISCUSSION

This study demonstrated the presence of OTR in MYO, LE, GE and stroma in the uterus of non-pregnant bitches. Derussi et al. (2012)

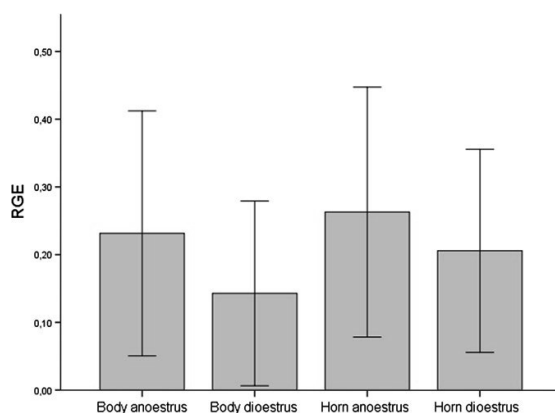


FIGURE 2 Relative expression of oxytocin receptor mRNA (mean \pm SD) in the non-pregnant canine uterus in the body and the horn, and during anoestrus and dioestrus. No statistically significant differences were detected. RGE: relative gene expression

reported the expression of OTR in the middle horn of non-pregnant bitches. In our study, in addition to middle horn, the expression of OTR was also seen in the body. There was no difference in expression

between the middle horn and the body. The samples in this study were of full thickness and it would be necessary for future studies to separate the longitudinal and circular muscular layers to compare the expression, as in the binding studies by Kitazawa et al. (2001), there was a different distribution of the OTR depending on the layer of the muscle and site of the porcine uterus. However, no significant difference was detected between uterine middle horn and body, or between circular and longitudinal layers of myometrium, in staining intensity for OTR in IHC. In the LE, the staining intensity was significantly higher in dioestrous than in anoestrous uteri. In early dioestrus, the uterus of a non-pregnant bitch eliminates possible infection after uterine bacterial invasion during cervical opening in oestrus. Typical time for pyometra to occur is during dioestrus, when uterus is under the influence of progesterone. Local endometrial paracrine or autocrine signalling of OT and OTR system may have a role in this cleansing process, preventing pyometra to develop. The possible role of LE and OTR system in pyometra should be studied further. The paracrine or autocrine signalling may also be involved in the implantation of embryos. Unlike for many other species, endometrial expression of OTR in the canine uterus probably has no influence on luteolysis because it has been shown that hysterectomy does not extend the luteal phase (Hoffmann et al., 1992).

The expression of OTR mRNA in the uterus of non-pregnant bitches during anoestrus and dioestrus supports the assumption that OT has a role in contracting activity also in these phases, not only during parturition. Ibuki, Haga, Muramatsu, Mizumoto, and Itoh (1997) described uterine contractions in non-pregnant bitches during anoestrus and dioestrus, and administration of OT stimulated the contractile activity. In vitro, oxytocin-induced contractions are more evident in myometrial longitudinal than circular fibres in the uterus of non-pregnant bitches (Gogny et al., 2010).

The progesterone levels are similar in pregnant and non-pregnant bitches, except for the more sudden drop of the level in pregnant ones before parturition (Concannon et al., 1975). In normal and aglepristone-induced parturition, the relative expression of OTR is upregulated (Gram et al., 2014). The absence of progesterone as such cannot be the only stimulation for OTR expression, because in anoestrus, the basal level of progesterone did not upregulate the OTR. Oestrogens increase uterine OTR expression in many species (Beard & Lamming, 1994; Richard & Zingg, 1990; Zingg & Laporte, 2003). In the pregnant bitches, the oestradiol levels drop simultaneously to progesterone (Hoffmann et al., 1994; Onclin et al. 2002) and cannot thus explain the prepartum increase in OTR. The influence of other hormones, such as relaxin, prolactin and prostaglandins to OTR expression, should be investigated.

Oxytocin receptors were present in vascular endothelial cells as also previously reported in canine (Gram et al., 2014) and ovine (Wu et al., 1996) uterus. Oxytocin has been reported to cause relaxation or contractions depending on the type of the blood vessel in dogs (Katusic, Shepherd, & Vanhoutte, 1986). In addition to providing nutrients to the uterus, the vascular network has role in hormonal signalling.

The neutrophil granulocytes were positively stained for OTR in IHC in this study. In bovine, T lymphocytes express OTR mRNA, and oestradiol and progesterone play a role in the regulation of this

expression (Ndiaye, Poole, & Pate, 2008). There is some evidence that OT decreases inflammatory processes, possibly by antioxidant action, increased endogenous glucocorticoids and diminished neutrophil infiltration (Biyikli et al., 2006; Işeri et al., 2005; Petersson, Wiberg, Lundeborg, & Uvnäs-Moberg, 2001).

In conclusion, despite the apparent inactivity, the uterus of the non-pregnant bitch expresses OTR. The distribution or relative expression of OTR does not differ between uterine horn and body in dioestrus or anoestrus except in LE. LE may have more oxytocin-dependent activity during dioestrus than anoestrus. For further studies, comparison of the cervix and the tip of the uterus, separate layers of myometrium, as well as more detailed stages of the whole reproductive cycle, especially at the time of luteal regression, should be included to clarify the action of OT and OTR system in the non-pregnant bitch.

ACKNOWLEDGEMENTS

Authors thank Antti Flyckt, Kati Holmsten, Reijo Jokivuori, Jere Linden, Merja Pöytäkangas and Satu Sankari from the University of Helsinki for their assistance in laboratory analyses and the staff of the Veterinary Teaching Hospital of the University of Helsinki, Saari Unit, for helping in the collection of the samples. Mariana Hulchiy from Karolinska Institutet is also acknowledged for her assistance in scoring the IHC. This study was financially supported by the Finnish Foundation of Veterinary Research, the Finnish Veterinary Foundation and the European Veterinary Society for Small Animal Reproduction (EVSSAR).

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHOR CONTRIBUTIONS

TMT designed the study and involved in RNA isolation, IHC, real-time PCR, data and statistical evaluation and manuscript writing. OV, JT and TK took part in design of the study, data and statistical evaluation and manuscript editing. LS and BM involved in RNA isolation, real-time PCR and IHC.

REFERENCES

- Beard, A. P., & Lamming, G. E. (1994). Oestradiol concentration and the development of the uterine oxytocin receptor and oxytocin-induced PGF2 alpha release in ewes. *Journal of Reproduction and Fertility*, 100, 469–475.
- Bhatti, S. F., Rao, N. A., Okkens, A. C., Mol, J. A., Duchateau, L., Ducatelle, R., van den Ingh, T. S., Tshamala, M., Van Ham, L. M., Coryn, M., Rijnberk, A., & Kooistra, H. S. (2007). Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch. *Domestic Animal Endocrinology*, 33, 294–312.
- Biyikli, N. K., Tuğtepe, H., Sener, G., Veliöğlu-Oğünç, A., Cetinel, S., Midilloğlu, S., Gedik, N., & Yeğen, B. C. (2006). Oxytocin alleviates oxidative renal injury in pyelonephritic rats via a neutrophil-dependent mechanism. *Peptides*, 27, 2249–2257.

- Christie, D. W., Bailey, J. B., & Bell, E. T. (1972). Classification of cell types in vaginal smears during the canine estrus cycle. *British Veterinary Journal*, 128, 301–310.
- Concannon, P. W., Hansel, W., & Visek, W. J. (1975). The ovarian cycle of the bitch: Plasma estrogen, LH and progesterone. *Biology of Reproduction*, 13, 112–121.
- Derussi, A. A., de Souza, R. W., Volpato, R., Guaitolini, C. R., Ackermann, C. L., Taffarel, M. O., Cardoso, G. S., Dal-Pai-Silva, M., & Lopes, M. D. (2012). Progesterone (PR), oestrogen (ER-alpha and ER-beta) and oxytocin (OTR) gene expression in the oviduct and uterus of pregnant and non-pregnant bitches. *Reproduction in Domestic Animals*, 47(Suppl 6), 197–199.
- Gimpl, G., & Fahrenholz, F. (2001). The oxytocin receptor system: Structure, function, and regulation. *Physiological Reviews*, 81, 629–683.
- Gogny, A., Mallem, Y., Destrumelle, S., Thorin, C., Desfontis, J. C., Gogny, M., & Fiéni, F. (2010). In vitro comparison of myometrial contractility induced by aglepristone-oxytocin and aglepristone-PGF2alpha combinations at different stages of the estrus cycle in the bitch. *Theriogenology*, 74, 1531–1538.
- Gram, A., Boos, A., & Kowalewski, M. P. (2014). Uterine and placental expression of canine oxytocin receptor during pregnancy and normal and induced parturition. *Reproduction in Domestic Animals*, 49(Suppl 2), 41–49.
- Hoffmann, B., Höveler, R., Hasan, S. H., & Failing, K. (1992). Ovarian and pituitary function in dogs after hysterectomy. *Journal of Reproduction and Fertility*, 96, 837–845.
- Hoffmann, B., Höveler, R., Nohr, B., & Hasan, S. H. (1994). Investigations on hormonal changes around parturition in the dog and the occurrence of pregnancy-specific non conjugated oestrogens. *Experimental and Clinical Endocrinology & Diabetes*, 102, 185–189.
- Ibuki, R., Haga, N., Muramatsu, S., Mizumoto, A., & Itoh, Z. (1997). Long-term observations of uterine contractions in nonpregnant dogs. *Biology of Reproduction*, 56, 632–639.
- Isaksson, E., Wang, H., Sahlin, L., von Schoultz, B., Cline, J. M., & von Schoultz, E. (2003). Effects of long-term HRT and tamoxifen on the expression of progesterone receptors A and B in breast tissue from surgically postmenopausal cynomolgus macaques. *Breast Cancer Research and Treatment*, 79, 233–239.
- Işeri, S. O., Sener, G., Sağlam, B., Gedik, N., Ercan, F., & Yeğen, B. C. (2005). Oxytocin ameliorates oxidative colonic inflammation by a neutrophil-dependent mechanism. *Peptides*, 26, 483–491.
- Katusic, Z. S., Shepherd, J. T., & Vanhoutte, P. M. (1986). Oxytocin causes endothelium-dependent relaxations of canine basilar arteries by activating V1-vasopressinergic receptors. *Journal of Pharmacology and Experimental Therapeutics*, 236, 166–170.
- Kitazawa, T., Kajiwara, T., Kiuchi, A., Hatakeyama, H., & Taneike, T. (2001). Muscle layer- and region-dependent distributions of oxytocin receptors in the porcine myometrium. *Peptides*, 22, 963–974.
- Kowalewski, M. P. (2014). Luteal regression vs. prepartum luteolysis: Regulatory mechanisms governing canine corpus luteum function. *Reproductive Biology*, 14, 89–102.
- Ndiaye, K., Poole, D. H., & Pate, J. L. (2008). Expression and regulation of functional oxytocin receptors in bovine T lymphocytes. *Biology of Reproduction*, 78, 786–793.
- Niswender, G. D., Juengel, J. L., Silva, P. J., Rollyson, M. K., & McIntush, E. W. (2000). Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews*, 80, 1–29.
- Onclin, K., Murphy, B., & Verstegen, J. P. (2002). Comparisons of estradiol, LH and FSH patterns in pregnant and nonpregnant beagle bitches. *Theriogenology*, 57, 1957–1972.
- Petersson, M., Wiberg, U., Lundeborg, T., & Uvnäs-Moberg, K. (2001). Oxytocin decreases carrageenan induced inflammation in rats. *Peptides*, 22, 1479–1484.
- Richard, S., & Zingg, H. H. (1990). The human oxytocin gene promoter is regulated by estrogens. *Journal of Biological Chemistry*, 265, 6098–6103.
- Robinson, R. S., Mann, G. E., Lammings, G. E., & Wathes, D. C. (1999). The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. *Journal of Endocrinology*, 160, 21–33.
- Robinson, R. S., Mann, G. E., Lammings, G. E., & Wathes, D. C. (2001). Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. *Reproduction*, 122, 965–979.
- Silva, E., Leitão, S., Ferreira-Dias, G., Lopes da Costa, L., & Mateus, L. (2009). Prostaglandin synthesis genes are differentially transcribed in normal and pyometra endometria of bitches. *Reproduction in Domestic Animals*, 44(Suppl 2), 200–203.
- Skarzynski, D. J., & Okuda, K. (1999). Sensitivity of bovine corpora lutea to prostaglandin F2alpha is dependent on progesterone, oxytocin, and prostaglandins. *Biology of Reproduction*, 60, 1292–1298.
- Spencer, T. E., Johnson, G. A., Burghardt, R. C., & Bazer, F. W. (2004). Progesterone and placental hormone actions on the uterus: Insights from domestic animals. *Biology of Reproduction*, 71, 2–10.
- Starbuck, G. R., Stout, T. A., Lammings, G. E., Allen, W. R., & Flint, A. P. (1998). Endometrial oxytocin receptor and uterine prostaglandin secretion in mares during the oestrous cycle and early pregnancy. *Journal of Reproduction and Fertility*, 113, 173–179.
- Wu, W. X., Verbalis, J. G., Hoffman, G. E., Derks, J. B., & Nathanielsz, P. W. (1996). Characterization of oxytocin receptor expression and distribution in the pregnant sheep uterus. *Endocrinology*, 137, 722–728.
- Zingg, H. H., & Laporte, S. A. (2003). The oxytocin receptor. *Trends in Endocrinology and Metabolism*, 14, 222–227.



Expression of uterine oxytocin receptors and blood progesterone, 13,14-dihydro-15-Keto-Prostaglandin $F_{2\alpha}$, and ionized calcium levels in dystocic bitches

Tuire Tamminen ^{a,*}, Lena Sahlin ^b, Britt Masironi-Malm ^b, Merja Dahlbom ^c, Terttu Katila ^a, Juhani Taponen ^a, Outi Laitinen-Vapaavuori ^d

^a Department of Production Animal Medicine, University of Helsinki, Paroninkuja 20, 04920, Saarentaus, Finland

^b Department of Women's and Children's Health, Karolinska Institutet and Karolinska University Hospital, 171 64, Solna, Sweden

^c Veterinary Clinic of Mäntsälä, Mäntymäentie 3, 04600, Mäntsälä, Finland

^d Department of Equine and Small Animal Medicine, University of Helsinki, Viikintie 49, 00790, Helsinki, Finland

ARTICLE INFO

Article history:

Received 4 February 2019

Received in revised form

2 June 2019

Accepted 5 June 2019

Available online 6 June 2019

Keywords:

Canine

Uterus

Birth

Progesterone

Prostaglandin $F_{2\alpha}$

ABSTRACT

This study aimed to examine the etiology of canine dystocia by measuring the relative expression of oxytocin receptor (OXTR) mRNA and the concentration of serum progesterone, plasma PGF_{2α} metabolite (PGFM), and blood ionized calcium (iCa) near term and in dystocia. Altogether 58 bitches were included in this study, 41 of which underwent cesarean section (CS). The four CS groups were based on history: complete uterine inertia (CUI; n = 7), partial uterine inertia (PUI; n = 13), obstructive dystocia (OD; n = 10), and elective cesarean section (ECS; n = 11). An additional group of medically treated dystocia without CS (MD; n = 8) and a control group (C; n = 9) with normal parturition (without CS and medical treatment) were also formed. Blood samples were taken prior to CS or medical treatment. Progesterone concentrations were highest in the ECS and a significant difference ($p < 0.05$) was observed between the ECS and the OD and between the ECS and the combined dystocia (CUI, PUI, OD, MD) groups (COMB). Highest concentrations of PGFM was observed in the C, the difference being significant ($p < 0.05$) between the C and the ECS and between the C and the COMB group. The progesterone:PGFM ratio was significantly ($p < 0.05$) higher in the ECS than in the C and the COMB group. No significant difference ($p > 0.05$) was observed in iCa concentrations between the groups. Relative OXTR mRNA expression was evaluated with real-time PCR from full-thickness uterine samples taken from the incision site during CS. The expression was highest in the ECS and the difference in expression was significant ($p < 0.05$) between the ECS and the OD and between ECS and the combined dystocia (CUI, PUI, OD) groups (COMB2). The study supports previous reports of decreasing progesterone and increasing PGFM during prepartum luteolysis. Upregulation of OXTR occurs near term. In obstructive dystocia, a prolonged influence of oxytocin and uterine exhaustion may lead to downregulation of OXTR. Complete primary uterine inertia may have a different etiology as no clear decrease in OXTR was observed in CUI as in OD. It remains unclear if parturition ceases because of uterine inertia or if uterine inertia occurs because of ceased parturition and desensitization of receptors.

© 2019 Elsevier Inc. All rights reserved.

1. Introduction

Parturition is a complex event and includes hormonal and behavioral changes, neural activity, and interaction between the dam and the offspring. Near term, canine plasma PGF_{2α} levels

increase leading to luteolysis, followed by a decrease in peripheral plasma progesterone levels [1–3] that allow for contractions of the uterus and for parturition to proceed [1]. The secretion of PGF_{2α} in the bitch is suggested to originate from placental trophoblast cells [4]. During parturition, increase in peripheral plasma cortisol [2,5,6], vasopressin [6], and oxytocin (OT) [6,7] occur. However, changes in cortisol levels vary greatly between individuals during parturition [2,5,6]. While estrogen concentrations are somewhat higher in the last trimester of pregnancy in the bitch, there is no

* Corresponding author.

E-mail address: tuire.tamminen@helsinki.fi (T. Tamminen).

marked prepartum increase as detected in many other species [8–10]. Two days prior to parturition, estrogen levels of the bitch decrease suddenly during prepartum luteolysis indicating its luteal source [11].

Occurrence of dystocia in bitches varies greatly depending on the population studied; the average is estimated to be below 5% [12]. In a group of 200 000 insured bitches (excluding Boston Terrier, English Bulldog, and French Bulldog) in Sweden, dystocia occurred in 16% of parturitions [13]. In the UK, the occurrence varied from 0% to 92% among 151 breeds (22, 005 litters) [14]. While dystocia seems to be more common in miniature and small breeds [13,15,16], several medium- and large-size breeds also have a higher than average proportion of litters born by cesarean section (CS) [14]. Approximately 60% of dystocia cases undergo CS [13,15–17]. In brachycephalic breeds, the proportion of CS is very high [14,18]. There may be a risk of bias in statistics of dystocia in these breeds due to the popularity of elective CS (ECS).

Dystocia is sometimes difficult to diagnose. Therefore, a complete history and physical examination is required. The suggested causes vary slightly according to different authors [12,19,20]. There are several, sometimes simultaneous, causes of dystocia. Maternal factors are more common than fetal factors. The most common maternal cause is primary uterine inertia, which can be complete or partial [12]. In complete primary uterine inertia, the uterus fails to initiate parturition due to absence of uterine contractions and thus no puppies are born [12,17]. In partial primary uterine inertia, the bitch may have weak uterine contractions or contractions that cease without any obvious reason (such as obstruction) before all puppies are born [12,17]. Secondary uterine inertia is caused by prolonged parturition due to obstruction in the birth canal [12,17].

Oxytocin is a nonapeptide hormone produced mainly in the hypothalamus and stored in the posterior pituitary gland. Oxytocin is released after suitable stimulus, such as intracervical pressure. As one of the most potent uterotonic hormones, OT enhances the contractility of the uterus. During parturition, plasma OT concentration increases [6,7]; this may not occur in dystocia [21,22]. The effect of OT in the uterus is mediated through specific, class I G-protein-coupled transmembrane receptors known as oxytocin receptors (OXTR) [23]. Near term, during prepartum luteolysis, OXTR are upregulated [24–26]. In humans, continuous exposure to OT leads to desensitization of OXTR by reduction of OT binding sites in the myometrial cell membrane and by downregulation of OXTR mRNA in myometrial cells [27]. While desensitization may also have a role in canine dystocia due to prolonged influence of OT, there is no published evidence of OXTR desensitization in bitches. The aim of this study was to examine the relative expression of OXTR mRNA in the canine uterus near parturition and in dystocia. Levels of serum progesterone, plasma prostaglandin F_{2α} metabolite (PGFM), progesterone:PGFM ratio, and blood ionized calcium (iCa) were also analyzed to clarify possible causative factors for dystocia.

2. Materials and methods

The study was approved by the Research Ethics Committee of the Viikki Campus, University of Helsinki, Finland. Blood sampling from bitches with normal parturition was authorized by the National Animal Experiment Board (ESAVI, Hämeenlinna, Finland), license number ESAVI/3802/04.10.03/2011.

2.1. Groups

Client-owned pet bitches that had CS performed either at the Small Animal Clinic of Mäntsälä or the Veterinary Teaching Hospital of the University of Helsinki were enrolled in the study (Table 1). The inclusion criteria were a diagnosis of dystocia resulting in CS or

ECS due to small litter size or previous dystocia. In addition, one group was established from bitches with medically treated mild dystocia that gave birth without CS. Bitches with normal parturitions served as controls for blood parameters. The owners of the bitches were requested to sign a written consent and complete a questionnaire to obtain the history of the bitch including previous and present parturitions. Any systemic disease was an exclusion criterion.

The following study groups were formed: 1) complete primary uterine inertia (CUI; *n* = 7, no puppies born, parturition does not proceed, discharge of fetal fluids >3 h or green discharge, no response to vaginal stimulus), 2) partial primary uterine inertia (PUI; *n* = 13, at least one puppy born, parturition ceases without obstruction), 3) obstructive dystocia (OD; *n* = 10, fetal oversize/narrow birth canal, malpresentation, malformation), 4) elective cesarean section (ECS; *n* = 11, 58–66 days from mating, previous dystocia, one or two puppies, before the onset of the stage 1 of parturition), 5) medically treated dystocia (MD; *n* = 8, no CS, medical treatment), 6) control (C; *n* = 9, no CS, no medical treatment, normal parturition). Dystocia groups were also combined (COMB: CUI, PUI, OD, MD and COMB2: CUI, PUI, OD) to compare with ECS and C. The diagnosis and treatment decisions were performed by the veterinarian on call. After blood sampling, the bitches were treated, if necessary, with calcium glubionate (Calcium-Sandoz®, Sandoz A/S, Copenhagen, Denmark) and oxytocin (Vetox®, Vetcare, Salo, Finland) (Table 1).

The individual and average data of the bitches are presented in Table 1. Altogether 35 different breeds were included in the study. Four bitches in the ECS group had had previous history of dystocia and CS. In the PUI and MD groups, each had one bitch with mild, medically treated dystocia without CS in the previous pregnancy. The previous parturitions of the other multiparous bitches were normal. The gestation length was calculated from ovulation day (at progesterone level 16–32 nmol/l) and from the first and the last mating according to the available information (Table 1).

2.2. Blood sampling

Blood samples were taken prior to CS or medical treatment from the vena cephalica into a syringe (Radiometer Safe Pico, ref: 956–610, Radiometer Medical, Copenhagen, Denmark), to an EDTA tube (Vacuette®, Mekalasi Oy, Nurmijärvi, Finland) with 5000 KIU aprotinin/ml EDTA blood (Aprotinin, Roche Diagnostics GmbH, Mannheim, Germany) and a serum tube with clotting activator (Vacuette®, Mekalasi Oy, Nurmijärvi, Finland). Blood samples were taken prepartum in the ECS group and peripartum (second stage of parturition) in the other groups. EDTA tubes and syringes were stored in an ice-water bath and serum tubes at room temperature. Blood samples were centrifuged (Eppendorf Centrifuge 5810R, Eppendorf Nordic A/S, Hørsholm, Denmark) as follows: EDTA tubes at 4 °C, 1200×*g*, 10 min, and serum tubes at 22 °C, 1700×*g*, 10 min. Plasma and serum were divided into aliquots, frozen at –20 °C, and stored at –70 °C until analyzed.

2.3. Progesterone assay

Serum progesterone concentrations were measured in one run using a commercial RIA kit (Progesterone Coat-A-Count® RIA, Siemens Healthcare Diagnostics Oy, Espoo, Finland) according to the manufacturer's instructions. The concentrations were measured in duplicate with a gamma counter (1272 GliniGamma, LKB Wallac Oy, Turku, Finland). The intra-assay coefficient of variation was 3.4% at a serum concentration of 4.4 nmol/L and 2.0% at a concentration of 32.5 nmol/L.

Table 1
The individual and average data of 58 bitches divided in six groups.

Group	Mean age, min-max (years)	Mean weight, min-max (kg)	Mean litter size, min-max	Breed	Parity	Gestation length (days from the last and first mating)	Gestation length (days from ovulation)	Duration of the first stage of parturition (hours)	Duration of the second stage of parturition before intervention (hours), discharge	Litter size	Number of puppies born before dystocia/ by cesarean section	Medication
1. Complete primary uterine inertia (CUI) n = 7	4.1, 2.3–6.2	23.7, 4.7–35.0	6.4, 1.0–14.0	Border Collie	1	56–61	n/a	26	3, fetal fluids	4	0/4	1
				Alredale Terrier	1	60–62	63	45	6, fetal fluids	7	0/7	0
				Karelian Bear Dog	3	68	n/a	n/a	n/a, green fluids	1	0/1	0
				Dogo Argentino	1	65	n/a	29	6, green fluids	2	0/2	0
				Chinese Crested	1	62	n/a	16	4, fetal fluids	6	0/6	1
				Dog								
				English Springer Sp.	2	60	n/a	48	6, fetal fluids	14	0/14	1
				Labrador Retriever	1	59	60	65	6, fetal fluids	11	0/11	0
2. Partial primary uterine inertia (PUI) n = 13	5.1, 2.6–8.0	29.2, 6.5–65.0	7.0, 5.0–15.0	Mixed	1	64–65	n/a	n/a	4	5	1/4	1
				Labrador Retriever	2	60–62	62	5	4	8	5/3	1
				Dalmatian	3	63	63	6	4	6	1/5	0
				Poodle, Standard	1	61–62	62–63	7	9.5	5	3/2	0
				Labrador Retriever	2	63	n/a	10	5	8	6/2	1
				Miniature Schnauzer	1	60–61	n/a	24	3.5	7	1/6	1
				Tibetan mastiff	2	59–61	62	24	4	5	4/1	1
				Mixed	1	62	n/a	27	n/a	7	1/6	1
				Border Collie	1	57–61	n/a	22	5.5	6	4/2	1
				Bullmastiff	2	56–57	57	65	n/a, fetal fluids	15	1/14	0
				Great Dane	1	62	n/a	26	6	6	2/4	1
				Bearded Collie	1	54–56	58	14	5.5	8	4/4	1
				Giant Schnauzer	2	58	60	12	10	5	2/3	1
				Cairn Terrier	3	64	66	n/a	4	1	0/1	0
3. Obstructive dystocia (OD) n = 10	5.1, 2.3–8.3	23.7, 7.0–65.0	5.0, 1.0–9.0	Border Collie	3	59–63	63	n/a	n/a, green fluids	7	0/7	1
				Saluki	2	61	64	18	4	3	0/3	0
				Cavalier King Ch.	1	59–60	62	24	4	7	0/7	0
				Sp.								
				French Bulldog	1	61–63	n/a	9	6	3	0/3	0
				Dog de Bordeaux	2	65–67	n/a	11	4	9	0/9	1
				Smooth Collie	3	60–61	n/a	24	8	6	3/3	1
				Cavalier King Ch.	1	56–58	59	n/a	3	4	1/3	1
				Sp.								
				Spanish Mastiff	1	62	62	11	n/a, fetal fluids	3	0/3	1
				Border Collie	3	60–61	61	24	3	7	0/7	1
				Cavalier King Ch.	3	n/a	60	0	0	4	0/4	0
4. Elective cesarean section (ECS) n = 11	4.6, 3.1–6.6	23.9, 6.9–65.0	3.8, 1.0–10.0	Sp.								
				Border Collie	1	64–65	64	0	0	2	0/2	0
				St. Bernard	2	n/a	61	0	0	2	0/2	0
				Cavalier King Ch.	3	58–60	60	0	0	5	0/5	0
				Sp.								
				Great Dane	2	62–63	62–63	0	0	1	0/1	0
				Shetland Sheepdog	4	60	n/a	0	0	1	0/1	0
				Boston Terrier	2	63–63	62	0	0	2	0/2	0
				Newfoundland	1	58–64	n/a	0	0	10	0/10	0
				Cavalier King Ch.	3	59–60	59	0	0	8	0/8	0
				Sp.								
				Boston Terrier	1	63–65	n/a	0	0	3	0/3	0
				Golden Retriever	1	64–66	65	0	0	4	0/4	0
				Dachshund	1	58–60	n/a	1	2	4	0/-	1

5.	Medically treated dystocia, no cesarean section (MD) n = 8	4.7, 2.6–6.5	13.3, 4.5–20.0	5.1, 2.0–8.0	Dachshund, miniature	1	63	n/a	15	5	6	0/-	1
					Finnish Lapphund	1	n/a	n/a	n/a	7	5	4/-	1
					Jack Russell Terrier	2	58–61	n/a	n/a	4	2	0/-	1
					Australian Kelpie	1	60	n/a	15	7	8	6/-	1
					English Springer Sp.	3	n/a	n/a	12	10	4	2/-	1
					Finnish Lapphund	2	n/a	n/a	n/a	5	6	3/-	1
					Lapponian Herder	2	n/a	63	7	5	6	2/-	1
					Cavalier King Ch.	1	n/a	60	n/a	n/a	5	—	0
6.	Control, normal parturition (C) n = 9	4.20, 1.9–6.5	10.9, 8.0–30.0	5.4, 4.0–10.0	Sp.	3	n/a	60	n/a	n/a	10	—	0
					Labrador Retriever	3	n/a	59	n/a	n/a	4	—	0
					Cavalier King Ch.	4	n/a	61–62	n/a	n/a	5	—	0
					Pug	2	n/a	61	n/a	n/a	5	—	0
					Pug	1	n/a	n/a	n/a	n/a	5	—	0
					Cavalier King Ch.	4	61	n/a	n/a	n/a	5	—	0
					Sp.	1	62	n/a	n/a	n/a	4	—	0
					Cavalier King Ch.	2	n/a	58–59	n/a	n/a	5	—	0
					Sp.	2	n/a	60	n/a	n/a	6	—	0
					Cavalier King Ch.	2	n/a	60	n/a	n/a	6	—	0

Parity includes the current parturition. Medication: 0, no medication; 1, calcium and oxytocin after blood sample. n/a: not available.

2.4. PGF_{2α} metabolite assay

Concentrations of the major metabolite of PGF_{2α}, 13,14-dihydro-15-keto-prostaglandin F_{2α} (PGFM), were measured from plasma using a commercial immunoassay kit (DetectX® 13,14-dihydro-15-keto-PGF_{2α} (PGFM) Enzyme Immunoassay Kit, Arbor Assays, Michigan, USA) according to the manufacturer's instructions. Prior to performing the assay, plasma samples were diluted 1:15 with the assay buffer provided in the kit. The optical density of each well was measured with a Multiscan GO Spectrophotometer with SkanIt software 4.1 (Thermo Fisher Scientific Oy, Vantaa, Finland). The intra-assay coefficient of variation of duplicates was 12.0%. The inter-assay coefficient of variation was 13.0% at a plasma concentration of 15.8 nmol/L and 3.5% at a concentration of 58.5 nmol/L. The linearity of the assay was evaluated by diluting the canine plasma sample (1/10, 1/20 and 1/40) with the assay buffer provided in the kit. Observed to expected ratios were calculated for the dilutions. The mean recovery of the expected PGFM concentrations in different dilutions was 102% and dilutions of the canine plasma sample showed linearity over the studied range ($R^2 = 0.996$). The detection limit was 0.13 nmol/L.

2.5. iCa

Blood iCa was analyzed instantly with Roche Electrolyte Analyzer (9180, Fisher Scientific Oy, Vantaa, Finland) from Safe Pico syringes stored in an ice-water bath. The syringes contained 60 IU of dry electrolyte-balanced heparin. Contact with air was minimized with a specific cap to remove possible air bubbles.

2.6. Uterine samples

Uterine samples were obtained only from bitches undergoing a CS. Immediately after the removal of the puppies from the uterus, a full-thickness sample of uterine wall (approximately 5×30 mm) was taken from the incision site (interplacental area, uterine body or proximal horn). The sample was immediately frozen in liquid nitrogen and stored at -70°C for PCR analysis to measure the relative expression of OXTR mRNA.

2.7. PCR

2.7.1. RNA preparation and reverse transcription

The full procedure has been described previously [28]. In brief, a 2-μg aliquot of total RNA from each canine uterine sample was reverse transcribed at 37°C for 60 min in a final volume of 20 μL with a reaction mixture (Qiagen) containing $1 \times$ RT buffer, dNTP mix (0.5 mM each dNTP), 600 ng random primers (Invitrogen, Paisley, UK), 2 units of RNase inhibitor (Qiagen), and 4 units of Omniscript™ reverse transcriptase (Qiagen).

2.7.2. Real-time PCR analysis

The real-time PCR analysis and the primers used have been described previously [28]. The oligonucleotide primer pair for the OXTR was designed with NCBI/Primer-BLAST. To standardize the quantification method, RPL27 and HPRT1 were selected as non-regulated reference genes with primer pairs obtained from Silva et al. [29] and Bhatti et al. [30], respectively. The primers were based on the sequences of the canine genes, and were the following: OTR forward primer: 5'-TGCTGGCCTTCATCGTGTGCT-3'; OTR reverse primer: 5'-GATGAAAGCCGAGGCTTCCTTGGG-3' from NM_001198659.1 with predicted size 95 bp; RPL27 forward primer: 5'-ACAATCACCTCATGCCACA-3'; RPL27 reverse primer: 5'-CTTGACCTGGCCTCTCGTC-3' from NM_001003102.2 with the predicted size 122 bp; HPRT1 forward primer: 5'-

AGCTTGCTGGTGAAAAGGAC-3'; HPRT1 reverse primer: 5'-TTA-TAGTCAAGGGCATATCC-3' from NM_001003357.1 with predicted size 104 bp. All samples were run in duplicate and the purity of PCR products was confirmed by a melting-curve analysis in all experiments. Each PCR assay included a negative control containing an RNA sample without reverse transcription. The PCR amplification rate and the cycle threshold (C_t) values were analyzed using iCycler™ iQ 3.1 software (Bio-Rad). The OXTR product was normalized against the mean of RPL27 and HPRT1 products to yield the relative expression of OXTR mRNA.

2.8. Statistical analysis

Data were analyzed using IBM SPSS Statistics 24 software for Windows. The non-parametric Kruskal-Wallis one-way ANOVA test with Bonferroni correction was used to detect possible differences in serum progesterone levels, plasma PGFM levels, blood iCa, and relative expression of OXTR mRNA between the groups. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Progesterone

Serum progesterone concentrations in the different groups are presented in Fig. 1a. The concentrations were highest in the ECS group; the largest variation in levels was also observed in this group. There was a significant difference ($p < 0.05$) between the ECS and the OD and between the ECS and the COMB groups.

3.2. PGFM

Plasma PGFM concentrations were highest in the C and lowest in the ECS group (Fig. 1b). A significant difference ($p < 0.05$) was detected between the C and the ECS and between the C and the COMB groups.

3.3. Progesterone:PGFM

The progesterone:PGFM ratio was highest in the ECS group (Fig. 1c). A significant difference ($p < 0.05$) was observed between the ECS and the C and between the ECS and the COMB groups.

3.4. iCa

Blood iCa concentrations were lowest in the PUI group but no significant difference ($p > 0.05$) was observed between the groups (Fig. 1d). No hypocalcemia was detected (reference interval 1.16–1.40 nmol/l).

3.5. qPCR

The mean relative expression of OXTR mRNA was highest in the ECS group (Fig. 1e). The difference was significant ($p < 0.05$) between the ECS and the OD and between the ECS and the COMB2 groups. There was no significant difference between bitches treated or not treated with calcium glubionate and OT.

4. Discussion

Our study indicates that in complete primary uterine inertia the etiology may not be the absence or downregulation of OXTR, as there was no difference in OXTR expression in comparison of CUI to bitches near term but before the first stage of parturition (ECS group). Upregulation of OXTR occurs near term, and the prolonged

influence of OT and uterine exhaustion in obstructive dystocia may lead to downregulation of OXTR.

Our results support previous reports [1–3] on decreasing progesterone and increasing PGFM levels during prepartum luteolysis in pregnant bitches. As expected, progesterone levels were higher in the ECS group than in the other groups, as CS was performed in this group before the onset of parturition (before stage 1). A sudden decrease of progesterone is observed in near term pregnant bitches at the end of the luteal phase [11]. Termination of corpora lutea function in non-pregnant bitches is suggested to be more likely regressive than the active luteolytic process found in pregnant bitches, which indicates a different regulation mechanism [31]. Failure of luteolysis can lead to prolonged gestation [32]. Except in the ECS group, all bitches in this study had undergone luteolysis.

One possibility for the etiology of complete primary uterine inertia could be a problem in parturition initiation. Excessive progesterone and insufficient PGF2 α levels could prevent sufficient uterine contractions and thus interfere with parturition. However, our results suggest that this might not be the case, as the progesterone and PGFM levels in CUI group were similar to other dystocia groups. This may indicate that the etiology is more likely at the level of uterine function, such as myometrial distention beyond its capacity to contract or the lack of cervical pressure to stimulate OT release. The progesterone:PGFM ratio was highest in the ECS group, where the highest progesterone and lowest PGFM concentrations were also found. This indicates that luteolysis had not yet occurred in this group. A high progesterone:PGFM ratio has been reported in dystocic bitches with complete primary uterine inertia in comparison to a control group [22]. In our study no such difference was observed.

Calcium and OT injections are used as a treatment for uterine inertia to enhance contractions of the uterus [19,20]. Batra [33] reported that OT-induced myometrial contractions in the rat depend on the influx of extracellular calcium, and this influx is directly increased by OT. The action of OT has also been postulated to occur by inhibiting the Ca²⁺-extrusion pump in humans [34]. Hypocalcemia was not diagnosed in any of the bitches in this study. However, there are reports of hypocalcemia in risk groups of uterine inertia [35] and in dystocic bitches [36]. In our study, a single treatment with calcium glubionate and OT did not seem to affect the expression of OXTR mRNA or distribution of OXTR. In dystocia, the uterus has been under the influence of OT, and exhaustion and desensitization may prevent medical treatment to induce uterine contractions. However, in this study the number of bitches treated or not treated was low and further investigation is necessary.

Veiga et al. [26] reported higher expression of OXTR mRNA in both endometrium and myometrium of late pregnant and parturient bitches than in earlier stages of pregnancy. In our study, full-thickness samples were used for real-time PCR; endometrium and myometrium thus cannot be compared separately. The samples of this study were run together with samples from our earlier report on non-pregnant bitches [28], and the relative expression of OXTR mRNA was higher in pregnant bitches than in non-pregnant ones. Expression of OXTR in the canine uterus is probably not regulated only by a decrease of progesterone. In anestrous bitches with basal levels of progesterone, OXTR expression does not differ from diestrous bitches with uteri under the influence of progesterone [28]; the expression is thus likely a part of more complex regulatory pathways. In the OD group, OXTR mRNA expression was significantly decreased. In the PUI group the decrease also approached significance. A large variation of OXTR mRNA expression in the CUI group may be due to the heterogeneity of this group. It is also possible that in the CUI group the mechanism of dystocia is different than that of the PUI and OD groups. The uterus does not

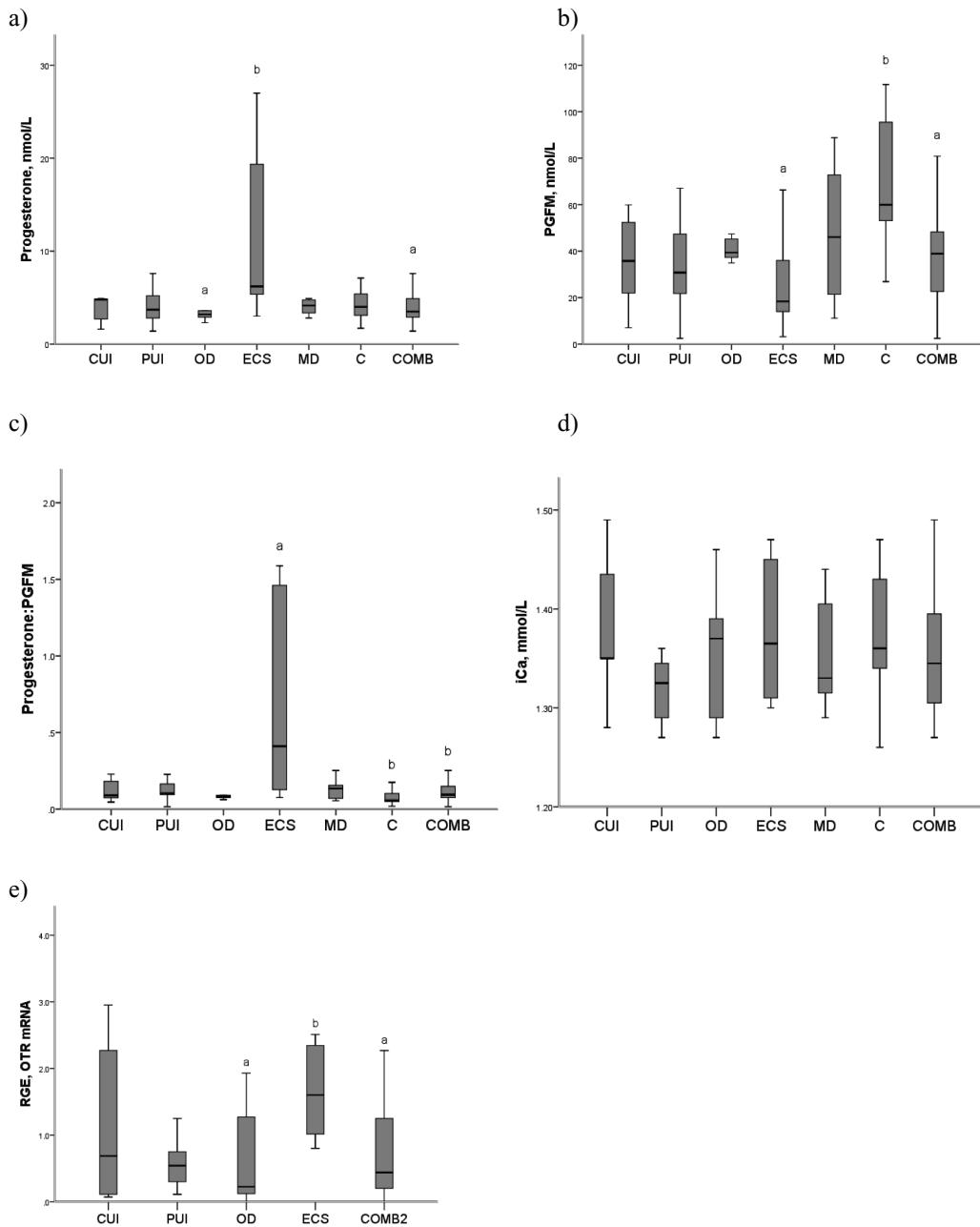


Fig. 1. Concentrations of hormones and ionized Ca (iCa), and relative gene expression (RGE) of uterine oxytocin receptor (OTR). a) serum progesterone, b) plasma prostaglandin $F_{2\alpha}$ metabolite (PGFM), c) progesterone:PGFM ratio, d) blood iCa, e) relative gene expression of OTR mRNA. The boxplots show the median with 50% of the data falling within the box. The whiskers extend to the 5th and 95th percentiles. Boxes with different letter designations are significantly different at $p < 0.05$. CUI, complete uterine inertia; PUI, partial uterine inertia; OD, obstructive dystocia; ECS, elective cesarean section; MD, medically treated dystocia; C, normal parturition; COMB, four dystocia groups combined: CUI, PUI, OD, MD; COMB2, three dystocia groups combined: CUI, PUI, OD.

contract in complete primary uterine inertia, which may be due to the lack of cervical stimulus and insufficient release of OT to systemic circulation. Thus, desensitization might not occur and expression of OXTR mRNA could remain high. Bergström et al. [21] reported lower plasma OT concentrations in primary uterine inertia cases than in bitches with normal parturition. In obstructive dystocia, and possibly in partial uterine inertia, uterine exhaustion possibly with paracrine or autocrine signaling may result in OXTR downregulation.

Although strict criteria were defined to include the bitches in the groups in this study, some heterogeneity probably exists. Breed diversity also increases the heterogeneity of the groups. In this study, the bitches with normal parturition were used as controls only for the blood parameters. For OXTR gene expression only ECS samples from prepartum bitches were used. Further studies are necessary to compare OXTR gene expression also with samples from bitches with normal parturition. The number of bitches was quite low (particularly in the CUI group), which may affect the results. A greater number of individuals is necessary to more properly evaluate the effect of calcium and OT treatment on OXTR. Furthermore, an uterokinet study *in vitro* with myometrial muscle strips, as described by Gogny et al. [37], may provide information on myometrial contractions and desensitization under prolonged influence of OT. Further studies of genetic background with breeds and lines susceptible to complete primary uterine inertia are needed.

5. Conclusions

This study provides evidence of prepartum upregulation of OXTR in the canine uterus. Expression of OXTR was increased near term. A decrease in expression was observed in obstructive dystocia and may also occur in partial primary uterine inertia. However, no clear decrease in expression was observed in the CUI group, which may indicate a different etiology for inertia than in OD. The etiology in complete primary uterine inertia is more likely at the level of uterine function, such as myometrial distention beyond its capacity to contract or the lack of cervical pressure to stimulate OT release. A decrease of OXTR may also occur during normal parturition; the role of desensitization of OXTR in dystocia should be clarified. It remains unclear if parturition ceases because of uterine inertia or if uterine inertia occurs because of ceased parturition and desensitization of receptors.

Author contributions

TMT: design of the study, RNA isolation, real-time PCR, data and statistical evaluation, manuscript writing. OV, MD, JT, TK: design of the study, data and statistical evaluation, manuscript editing. LS, BM: RNA isolation, real-time PCR, manuscript editing.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

The authors would like to thank the following from the University of Helsinki: Kati Holmsten, Merja Pöytäkanas, and Satu Sankari for assistance in laboratory analyses. The staff of the Veterinary Teaching Hospital of the University of Helsinki and the Veterinary Clinic of Mäntsälä is acknowledged for assistance in sample collection. This study was financially supported by the Finnish Foundation of Veterinary Research, the Finnish Veterinary Foundation, and the European Veterinary Society for Small Animal Reproduction (EVSSAR).

References

- [1] Concannon PW, Isaman L, Frank DA, Michel FJ, Currie WB. Elevated concentrations of 13,14-dihydro-15-keto-prostaglandin F-2 alpha in maternal plasma during prepartum luteolysis and parturition in dogs (*Canis familiaris*). *J Reprod Fertil* 1988;84:71–7.
- [2] Veronesi MC, Battocchio M, Marinelli L, Faustini M, Kindahl H, Cairoli F. Correlations among body temperature, plasma progesterone, cortisol and prostaglandin F2α of the periparturient bitch. *J Vet Med A* 2002;49:264–8.
- [3] Nohr B, Hoffmann B, Steinetz BE. Investigation of the endocrine control of parturition in the dog by application of an antigestagen. *J Reprod Fertil Suppl* 1993;47:542–3.
- [4] Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Küküçüoğlu I, et al. Canine placenta: a source of prepartal prostaglandins during normal and antiprogesterin-induced parturition. *Reproduction* 2010;139:655–64.
- [5] Concannon PW, Butler WR, Hansel W, Knight PJ, Hamilton JM. Parturition and lactation in the bitch: serum progesterone, cortisol and prolactin. *Biol Reprod* 1978;19:1113–8.
- [6] Olsson K, Bergström A, Kindahl H, Lagerstedt A-S. Increased plasma concentrations of vasopressin, oxytocin, cortisol and the prostaglandin F2α metabolite during labour in the dog. *Acta Physiol Scand* 2003;179:281–7.
- [7] Klarenbeek M, Okkens AC, Kooistra HS, Mol JA, Bevers MM, Taverne MAM. Plasma oxytocin concentrations during late pregnancy and parturition in the dog. *Theriogenology* 2007;68:1169–76.
- [8] Yoshinaga K, Hawkins RA, Stocker JF. Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. *Endocrinology* 1969;85:103–12.
- [9] Robertson HA, Smeaton TC. The concentration of unconjugated oestrone, oestradiol-17 alpha and oestradiol-17 beta in the maternal plasma of the pregnant Ewe in relation to the initiation of parturition and lactation. *J Reprod Fertil* 1973;35:461–8.
- [10] Robertson HA. Changes in the concentration of unconjugated oestrone, oestradiol-17alpha and oestradiol-17beta in the maternal plasma of the pregnant cow in relation to the initiation of parturition and lactation. *J Reprod Fertil* 1974;36:1–7.
- [11] Concannon PW, Hansel W, Visek WJ. The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. *Biol Reprod* 1975;13:112–21.
- [12] Linde-Forsberg C. Abnormalities in pregnancy, parturition, and the periparturient period. In: Ettinger SJ, Feldman EC, editors. *Textbook of veterinary internal medicine*. seventh ed. St. Louis: Elsevier Saunders; 2010. p. 1890–901.
- [13] Bergström A, Nødtvedt A, Lagerstedt AS, Egenvall A. Incidence and breed predilection for dystocia and risk factors for cesarean section in a Swedish population of insured dogs. *Vet Surg* 2006;35:786–91.
- [14] Evans KM, Adams VJ. Proportion of litters of purebred dogs born by caesarean section. *J Small Anim Pract* 2010;51:113–8.
- [15] Gaudet DA. Retrospective study of 128 cases of canine dystocia. *J Am Anim Hosp Assoc* 1985;21:813–8.
- [16] Münnich A, Küchenmeister U. Dystocia in numbers – evidence-based parameters for intervention in the dog: causes for dystocia and treatment recommendations. *Reprod Domest Anim* 2009;44(Suppl 2):141–7.
- [17] Darvelid AW, Linde-Forsberg C. Dystocia in the bitch: a retrospective study of 182 cases. *J Small Anim Pract* 1994;35:402–7.
- [18] Linde Forsberg C, Persson G. A survey of dystocia in the Boxer breed. *Acta Vet Scand* 2007;49:8.
- [19] Johnston SD, Root Kustritz MV, Olson PNS. Canine and feline theriogenology. first ed. Philadelphia: WB Saunders; 2001.
- [20] Davidson A. Problems during and after parturition. In: England G, von Heimendahl A, editors. *BSAVA Manual of canine and feline reproduction and neonatology*. Gloucester: British Small Animal Veterinary Association; 2010. p. 121–34.
- [21] Bergström A, Fransson B, Lagerstedt A-S, Olsson K. Primary uterine inertia in 27 bitches: aetiology and treatment. *J Small Anim Pract* 2006;47:456–60.
- [22] Bergström A, Fransson B, Lagerstedt AS, Kindahl H, Olsson U, Olsson K. Hormonal concentrations in bitches with primary uterine inertia. *Theriogenology* 2010;73:1068–75.
- [23] Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. *Physiol Rev* 2001;81:629–83.
- [24] Derussi AA, de Souza RW, Volpato R, Guaitolini CR, Ackermann CL, Taffarel MO, et al. Progesterone (PR), oestrogen (ER-alpha and ER-beta) and oxytocin (OTR) gene expression in the oviduct and uterus of pregnant and non-pregnant bitches. *Reprod Domest Anim* 2012;47(Suppl 6):197–9.
- [25] Gram A, Boos A, Kowalewski MP. Uterine and placental expression of canine oxytocin receptor during pregnancy and normal and induced parturition. *Reprod Domest Anim* 2014;49(Suppl 2):41–9.
- [26] Veiga GA, Milazzotto MP, Nichi M, Lúcio CF, Silva LC, Angriman DS, et al. Gene expression of estrogen and oxytocin receptors in the uterus of pregnant and parturient bitches. *Braz J Med Biol Res* 2015;48:339–43.
- [27] Phaneuf S, Asbóth G, Carrasco MP, Europe-Finner GN, Saji F, Kimura T, et al. The desensitization of oxytocin receptors in human myometrial cells is accompanied by down-regulation of oxytocin receptor messenger RNA. *J Endocrinol* 1997;154:7–18.
- [28] Tamminen TM, Sahlin L, Masironi B, Taponen J, Laitinen-Vapaavuori O, Katila T. Oxytocin receptors in dioestrous and anoestrous canine uteri. *Reprod*

- Domest Anim 2017;52:153–9.
- [29] Silva E, Leitão S, Ferreira-Dias G, Lopes da Costa L, Mateus L. Prostaglandin synthesis genes are differentially transcribed in normal and pyometra endometria of bitches. *Reprod Domest Anim* 2009;44(Suppl 2):200–3.
- [30] Bhatti SF, Rao NA, Okkens AC, Mol JA, Duchateau L, Ducatelle R, et al. Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch. *Domest Anim Endocrinol* 2007;33:294–312.
- [31] Kowalewski MP. Luteal regression vs. prepartum luteolysis: regulatory mechanisms governing canine corpus luteum function. *Reprod Biol* 2014;14: 89–102.
- [32] Irons PC, Nöthling JO, Volkmann DH. Failure of luteolysis leads to prolonged gestation in a bitch: a case report. *Theriogenology* 1997;48:353–9.
- [33] Batra S. Effect of oxytocin on calcium influx and efflux in the rat myometrium. *Eur J Pharmacol* 1986 Jan 14;120:57–61.
- [34] Popescu LM, Nutu O, Panoiu C. Oxytocin contracts the human uterus at term by inhibiting the myometrial Ca^{2+} -extrusion pump. *Biosci Rep* 1985;5:21–8.
- [35] Hollinshead FK, Hanlon DW, Gilbert RO, Verstegen JP, Krekeler N, Volkmann DH. Calcium, parathyroid hormone, oxytocin and pH profiles in the whelping bitch. *Theriogenology* 2010;73:1276–83.
- [36] Frehner BL, Reichler IM, Keller S, Goericke-Pesch S, Balogh O. Blood calcium, glucose and haematology profiles of parturient bitches diagnosed with uterine inertia or obstructive dystocia. *Reprod Domest Anim* 2018;53:680–7.
- [37] Gogny A, Mallem Y, Destrumelle S, Thorin C, Desfontis JC, Gogny M, et al. In vitro comparison of myometrial contractility induced by aglepristone-oxytocin and aglepristone-PGF2alpha combinations at different stages of the estrus cycle in the bitch. *Theriogenology* 2010;74:1531–8.

III

MONITORING OF UTERINE CONTRACTIONS IN THE BITCH

Tuire Tamminen^a, Terttu Katila^a, Outi Laitinen-Vapaavuori^b, Merja Dahlbom^c, Juhani Taponen^a

^aDepartment of Production Animal Medicine, University of Helsinki, Paroninkuja 20, FI-04920 Saarentaus, Finland

^bDepartment of Equine and Small Animal Medicine, University of Helsinki, Viikintie 49, FI-00790 Helsinki, Finland

^cVeterinary Clinic of Mäntsälä, Mäntymäentie 3, FI-04600 Mäntsälä, Finland

Corresponding author: Tuire Tamminen; Tel.: +358-50-4150196; E-mail: tuire.tamminen@helsinki.fi (T. Tamminen)

Abstract

Uterine inertia is a common reason for canine dystocia. Human cardiotocography (CTG) was tested in monitoring uterine contractions of bitches. Uterine contractions of 47 pregnant bitches representing 21 different breeds were monitored with CTG. The monitoring included all stages of normal parturition, i.e. prepartum (PRE, n = 13), first (FIRST, n = 7), and second (NORM, n = 17) stages. In addition, the following types of dystocia were incorporated: complete uterine inertia (CUI, n = 3), partial uterine inertia (PUI, n = 8), obstructive dystocia (OD, n = 3), and medically assisted dystocia (MD, n = 6). The monitoring times varied from 7 to 170 minutes depending on cooperation of the bitches. The relative change of pressure (amplitude change in contraction graph, %-units) was lowest in PRE, and a significant difference ($p < 0.05$) was noted between PRE and NORM, PRE and OD, and PRE and MD. The number of contractions in 10 minutes was lowest in PRE, and PRE was significantly different from NORM, PUI, and MD. The duration of contractions was shortest in PRE, and a significant difference was present between PRE and NORM and between PRE and MD. Fetal heartbeats were detected with the Doppler probe in 85% of cases, however, the method was sensitive to errors. Tocography is useful in diagnosis of uterine inertia and in monitoring response to uterotonic medicines, but it cannot replace careful clinical observation and examination.

KEYWORDS

Canine; dystocia; parturition; tocography; tocodynamometry

1 INTRODUCTION

Behavior of bitches during normal parturition and the duration of parturition vary [1,2]. Diagnosis of dystocia is sometimes challenging. Occurrence of dystocia in bitches has been estimated at under 5% [3] or around 16% [4], but in certain breeds the occurrence is markedly higher. Dystocia increases the risk of puppy mortality [5,6]. The most common maternal cause for dystocia is uterine inertia, which can be complete; the uterus fails to initiate parturition due to the absence of uterine contractions, and thus, no puppies are born [2,3]. Uterine inertia may also be partial; the bitch may have weak uterine contractions or contractions that cease without any obvious reason (such as obstruction) before all puppies are born [2,3]. Secondary uterine inertia is caused by prolonged parturition due to, for instance, obstruction in the birth canal [2,3]. Cesarean section is performed on approximately 60% of bitches with dystocia [4,5].

Follow-up of progress during parturition can be challenging. Some bitches show only minor changes in behavior, although parturition is not progressing and the litter is already lost, whereas others display signs of dystocia even though the parturition is proceeding well. To recognize parturitions where vitality of the puppies is at risk, an advanced method of parturition surveillance is necessary. With faster and more accurate diagnostic tools, puppy mortality might be reduced. Cardiotocography (CTG) could be an alternative to enhance monitoring of parturition in dogs. In the United States, Whelpwise™ Veterinary Perinatal Specialties® offers service and equipment for veterinarians and breeders to monitor uterine contractions and fetal heart rate before and during parturition at home. However, there are only a few reports on this method in dogs [7-9].

In women, CTG has been used for decades to monitor uterine contractions and fetal distress. The follow-up may be intermittent or continuous depending on the stage and risks of labor [10]. In human medicine, interpretation of CTG includes observation of fetal heart frequency comprising basal heart rate level, changes in the rate in the short and long term, and acceleration and deceleration of the rate [10]. The frequency, intensity, and duration of uterine contractions as well as changes in fetal heart rate in relation to uterine contractions are followed [10]. External monitoring of uterine contractions with CTG is not directly quantitative, but gives information on the relative strength, duration, and frequency of contractions. The frequency of contractions correlates well with methods of monitoring intrauterine pressure, but the strength and duration of contractions may be inaccurate, especially in obese human patients [10,11].

As dogs often behave restlessly during parturition [1,2] and may have long fur and have a uterus with long horns, several fetuses, and possibly uneven contractions of the horns [12], difficulties may occur in monitoring parturition using equipment intended for human medicine. The aim of this study was to test the suitability of two human extrauterine CTG devices in monitoring pre- and peripartum canine uterine contractions and fetal heartbeats.

2 MATERIALS AND METHODS

The study was approved by the Viikki Campus Research Ethics Committee, University of Helsinki, Finland. Oral consent for monitoring dogs was obtained from owners.

2.1. Animals and groups

Altogether 47 client-owned pregnant bitches were monitored at home or in a private veterinary clinic before the onset of parturition and during parturition. The weight of the bitches varied from 5 to 65 kg, the mean being 20.6 kg (± 15.1). The mean number of puppies was 6.1 (± 2.7). The breeds were as follows: Airedale Terrier (n=1), Australian Kelpie (n=1), Border Collie (n=3), Bullmastiff (n=1), Cavalier King Charles Spaniel (n=15), Doberman (n=1), English Springer Spaniel (n=1), Finnish Lapphund (n=2), Giant Schnauzer (n=3), Golden Retriever (n=1), Great Dane (n=1), Jack Russell Terrier (n=1), Labrador Retriever (n=5), Lapponian Herder (n=1), Miniature Poodle (n=1), Mixed (n=1), New Foundland Dog (n=2), Podengo Portuguese (n=1), Pug (n=2), Rough Collie (n=1), Spanish Mastiff (n=1), Tibetan Mastiff (n=1).

The monitors were also tested in five non-pregnant individuals to detect the effect of movement of the body to pressure changes. The breeds involved were two Border Collies, two Cavalier King Charles Spaniels, and one Miniature Poodle. Their weight varied from 7 to 21 kg, the mean being 12.8 kg (± 5.9).

The number of monitoring sessions of each bitch varied from 1 to 5. Monitoring time was intended to be 30 minutes a session, but it depended on the bitch's cooperation. Timing of prepartum monitoring varied from 55 to 61 days after ovulation and from 58 to 62 days from mating. Monitorings represented stages of normal parturition as well as different types of dystocia as follows: prepartum (PRE, n = 13), during the first (FIRST, n = 7) and second (NORM, n = 17) stages of normal parturition, in complete uterine inertia (CUI, n = 3), in partial uterine inertia (PUI, n = 8), in obstructive dystocia

(OD, $n = 3$), and in medically assisted dystocia (MD, $n = 6$). The criteria in dystocia groups were as follows: CUI, no puppies born, parturition does not proceed, discharge of fetal fluids > 3 hours or green discharge, no response to vaginal stimulus; PUI, at least one puppy born, parturition ceases without obstruction; OD, fetal oversize/narrow birth canal, malpresentation, malformation; MD, parturition assisted with medical treatment, no caesarean section.

The bitches were treated, if necessary, with oxytocin (Vetox®, Vetcare, Salo, Finland, 1-4 IU im). Monitorings were performed after oxytocin administration in 12 bitches (six in PUI, six in MD).

2.2. Cardiotocography (CTG) monitoring protocol

Two human CTG monitoring units (Sonicaid FM 820 and TeamCare, Algol Diagnostics, Vantaa, Finland) were used to detect uterine contractions and fetal heartbeats. In tocography, also known as tocodynamometry, uterine contractions are measured with a specific extrauterine probe. The method is based on measuring relative changes in pressure through abdominal muscles, skin, and hair. A Doppler probe, which records the fetal heartbeats, is connected to the CTG. Pressure changes and the fetal heart rate are shown electronically on a screen and printed as a graph. Here, the tocographic probe was placed caudal to the costal arch and lateral to the midline of the abdomen and fixed in place with a flexible belt (Fig. 1). At the onset of monitoring, the level of the pressure was zeroed to set the basal level (10% units). The reset to zero was repeated when necessary: if at the beginning the adjustment to zero took place during a uterine contraction or if the position or tightness of the belt was corrected later. Changes in the posture of the bitch were followed (probe under the bitch, the bitch sitting, standing, or moving). Also, general nervousness or relaxation was recorded (bitch relaxed, panting, or having nervous movements). Paper printing speed for tocographs was 1 or 2 cm/min. When printing was not possible because of a technical problem or paper had run out (13 monitoring sessions), the amplitude and frequency of contractions were recorded manually from the screen. The relative pressure change (amplitude changes above 10% units), and number, duration (> 1 minute), and interval of the contractions were counted. Occasionally, uterine activity below the limits set (amplitude change below 10% units, contraction duration < 1 minute) was noticed, but these were not counted. Fetal heartbeats were measured with a Doppler probe whenever possible. If necessary, the position of the probe was determined by localizing the heartbeats with a stethoscope. Hair was clipped when necessary and ultrasound gel was used. Both monitors were used in all groups and tocographic scale and patterns were similar. Thus, the results were combined.

2.3. Statistical analysis

The non-parametric Kruskal-Wallis one-way ANOVA test with Bonferroni correction (IBM SPSS Statistics 24 software for Windows) was used to detect differences between the groups in the relative pressure change (amplitude change in contraction graph, %-units), in the number of contractions per 10 minutes, and in the duration of the contractions. Differences were considered significant at $p < 0.05$.

3 RESULTS

Observed contractions are presented in Table 1. The three CUI bitches showed no contractions. The relative change of pressure (amplitude change) was lowest in PRE (10-15% units) and highest in NORM (20-45% units), and a significant difference ($p < 0.05$) emerged between PRE and NORM, PRE and OD, PRE and MD. The number of contractions in 10 minutes was lowest in PRE (0.7) and highest in OD (1.3) and MD (1.3), and a significant difference was detected between PRE and NORM, PRE and PUI, and PRE and MD. The mean duration of contractions was shortest in PRE (2.1 min) and longest in NORM (3.1 min), and a significant difference was present between PRE and NORM, and PRE and MD. The total number of contractions and the interval between contractions were not compared due to great variation in monitoring times. Selected contraction patterns are presented in Fig. 2. In PUI, six of eight bitches were treated with oxytocin before caesarean section; in five bitches a weak response (10-25% units amplitude change) was noted, and one bitch showed no response (no amplitude change). In MD, all six bitches were treated with oxytocin; five showed a strong response (25-45% units amplitude change) and one a weak response (15% units amplitude change), but this bitch gave birth (six puppies) without caesarean section.

Panting and movements of the bitch resulted in short-lasting sharp spikes in graphs. These spikes were also observed in non-pregnant individuals when they moved (amplitude change 10-20% units) but panting caused even higher spikes (amplitude change up to 90% units). A bitch lying on the probe for a while resulted in a similar curve as in uterine contraction, but with a higher amplitude change (40-70% units). In very restless bitches, monitoring was impossible. Four bitches were clearly uncomfortable with the probe and the belt around the abdomen; in these cases, the monitoring was discontinued. Two bitches tried to bite the belt even after adjustment of the position of the probe and loosening of the belt.

Fetal heartbeats were detected in 85% of cases. When the litter size was less than four, heartbeats were easier to recognize individually, whereas in large litters it was impossible. The most common factors for erroneous results were incorrect positioning of the probe, restless movements, and overweight of the dam. The probe readily recorded the maternal heartbeat instead of the fetal heartbeat. For clinical decisions, the fetal heart rate was ensured by ultrasonographic examination.

4 DISCUSSION

Uterine contractions and fetal heartbeats were detected with both monitors (Sonicaid FM 820 and TeamCare) tested. We were able to detect uterine inertia and follow the response to oxytocin treatment. Monitoring with CTG gives information about uterine functionality, especially in cases where uterine contractions are not clearly visible. Interpretation guidelines for CTG monitoring in bitches do not exist. It is challenging to define clear limits and criteria for normal and abnormal contraction patterns due to the great variety in the number of puppies, in the size of dams, and in the behavior of bitches during parturition.

In humans, according to the manuals of the tested CTG devices, the tocographic probe is positioned cranially to the navel or slightly laterally to the midline of the abdomen and kept in place with an elastic belt. The aim is to focus on the fundus of the uterus [13]. As the bitch has a bicornuate uterus, the position of the probe was not as accurate in our study but was situated somewhere in the middle of the horns. The surface of the probe is made of self-sticking plastic, which prevents the sliding of the probe from the desired position on human skin. This does not work in dogs, probably due to the lack of sweat glands in canine skin. A flexible belt was used to stabilize the position of the probe in this study. The position of the probe needed adjustment after expulsion of a puppy and sometimes after vigorous movements of the bitch.

We used a paper speed of 1 or 2 cm/min (Fig. 2). In humans, the speed of the paper is usually 1 cm/min, but 2 or 3 cm/min is also used, and it is recommended to use the speed with which the staff is most familiar [10]. The speed of the paper affects the pattern of the graph, and thus, may have an effect on the interpretation. In our study, however, we did not have interpretation difficulties associated with paper speed. The slower paper speed (1 cm/min) saves paper and seems to be adequate for detecting contractions in bitches.

According to our results, uterine contractions in bitches in normal parturition seem to commonly last longer than two minutes. In women, periparturient uterine contractions usually last from 45 to 120 seconds [10]. More than five contractions in 10 minutes in two 10-minute periods, or averaged over a 30-minute period, is considered in women as tachysystole, i.e. excessive frequency of uterine contractions [10,14]. There is no definition for canine tachysystole. This does not mean that tachysystole does not exist in bitches, but the criterion remains to be defined. In our study, none of the bitches had more than five contractions in 10 minutes. The number of contractions in 10 minutes is probably not the optimal way to describe contractility because bitches usually have multiple offspring, and the resting periods between deliveries of puppies may be long. Prepartum bitches had significantly less contractions in 10 minutes than NORM, PUI, and MD bitches, revealing that non-parturient bitches are easily distinguished from bitches in which parturition has already started. No difference was seen between dystocia groups, with the exception of CUI, where no contractions were noted. Thus, the diagnosis of uterine inertia was easy to set, and the necessary measures were undertaken in good time. A response to oxytocin administration was evident in tocography. Although the strength and duration of contractions are not considered unquestionably reliable with external CTG, we noticed that overall the contractions were weakest and shortest in prepartum bitches.

Fetal heartbeats were detected with CTG, but the method is sensitive to artifacts, and maternal heartbeats were easily recorded instead of fetal heartbeats, which is a recognized problem also in humans [15]. The canine fetus and its heart are smaller and the heart rate (160-220 bpm) [3] faster than in the full-term human fetus (110-160 bpm) [10]. These anatomical and physiological features of dogs may complicate the use of the method. Dogs usually have multiple offspring and it is impossible to follow heart rates of all fetuses. The caudal ones that are presented first to the birth canal are usually also the first ones in distress. Monitoring of fetal distress in dogs subsequent to uterine contraction would be of interest because uterine contractions reduce the placental blood flow, decreasing oxygen supply to the fetus [16,17]. We could not demonstrate fetal distress after uterine contractions with CTG in our study; it was confirmed with ultrasound examination. The monitoring method must be improved to advance diagnostics of fetal distress. A fetal scalp electrode is used in human medicine when external monitoring of fetal heartbeats is disturbed [10]. To our knowledge, this method has not been used in dogs, and it may be challenging due to the small size of the bitch, the long vagina, and the large number of fetuses. However, the high level of monitoring in humans may not be necessary in dogs.

In our study, the monitoring time was quite short on many occasions, and consequently, some contractions may have been missed. Shorter contractions, the ones below the one-minute limit, may also be relevant for progression of parturition. Asynchronous contractions in the two horns may also occur, which may lead to misinterpretations. Artifacts caused by movements of the bitch yield a graph with an intermittent curve and, in the worst case, may mask a uterine contraction. However, recognizing a contraction despite an intermittent curve is likely usually possible. Because a bitch lying on the probe results in a graph similar to a contraction, attention should be paid to the bitch's position.

The next step for the clinical use of CTG would be a more systematic follow-up of individuals. It is recommended that the clinical staff become well acquainted with the CTG device to be used to identify which paper speed yields graphs easy to interpret, what kind of artifacts to expect, and how to adjust the probe correctly. Hair clipping may help to keep the probe in place on dogs with a heavy coat, but clipping does not seem to be necessary to detect pressure changes. During long-term or even continuous monitorings the precision of contraction measurements could be increased, but it should be kept in mind that bitches may have long resting periods during normal parturition, as delivery of an entire litter can last up to 24 hours [2]. Follow-up of parturition should be done in cooperation with the breeder and fully trained animal hospital staff. The use of CTG should never replace careful clinical observation and examination and the use of other methods like ultrasonography when necessary.

The intrauterine pressure catheter is considered the most reliable method in uterine contraction monitoring [18]. To obtain more information on contraction patterns in the canine uterus, a study with simultaneous external and intrauterine monitoring, in both horns, with actual pressure measurement should be conducted. A more recent, non-invasive external method is electrohysterography (EHG), which records the electrical activity of the uterine muscle using surface electrodes applied to the maternal abdomen around the navel [13,19]. It correlates well with monitoring of intrauterine pressure and has proven to be more reliable than CTG, also in obese patients [13]. In mares, uterine contractions have been investigated by using transrectal ultrasound in B-mode [20] and M-mode [21]. EHG and ultrasound could be worth for testing in diagnosing canine uterine contractility. It remains to be clarified what kind of frequency of contractions is required for normal progression of parturition. Uterine contractility in specific clinical cases, such as uterine torsion or rupture, is also of interest. However, no typical pattern in tocography has been repeatedly reported for uterine rupture in human medicine [22]. Also, a protocol for monitoring fetal distress with CTG after uterine contraction should be devised.

5 CONCLUSIONS

The two monitors tested detected uterine contractions in the bitch. The monitors were easy to use, but were not readily portable, thus being best suited for hospital use, although many breeders managed to use the monitors successfully at home. It is important to recognize possible artifacts in tocographs and not to forget the clinical entity of the individual case. The method facilitated diagnosis of uterine inertia and monitoring of the response to oxytocin treatment.

ACKNOWLEDGMENTS

The authors thank the breeders, especially Jonna Laitinen, Kirsti Määttänen, and Anu Purola, for their participation. The study was financially supported by the Finnish Foundation of Veterinary Research and the Finnish Veterinary Foundation.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

TT: design of the study, patient monitoring, data evaluation, manuscript writing. MD, TK, OL-V, JT: data evaluation, manuscript editing.

REFERENCES

- [1] Bleicher N. Behavior of the bitch during parturition. *J Am Vet Med Assoc* 1962;140:1076-82.
- [2] Johnston SD, Root Kustritz MV, Olson PNS. *Canine and feline theriogenology*. 1st ed. Philadelphia: WB Saunders; 2001, p. 105-28.
- [3] Linde-Forsberg C. Abnormalities in pregnancy, parturition, and the periparturient period. In: Ettinger SJ, Feldman EC, editors. *Textbook of veterinary internal medicine*, 7th ed. St. Louis: Elsevier Saunders; 2010, p. 1890–901.

- [4] Bergström A, Nødtvedt A, Lagerstedt AS, Egenvall A. Incidence and breed predilection for dystocia and risk factors for cesarean section in a Swedish population of insured dogs. *Vet Surg* 2006;35:786-91.
- [5] Darvelid AW, Linde-Forsberg C. Dystocia in the bitch: A retrospective study of 182 cases. *J Small Anim Pract* 1994;35:402-7.
- [6] Münnich A, Küchenmeister U. Dystocia in numbers – Evidence-based parameters for intervention in the dog: Causes for dystocia and treatment recommendations. *Reprod Dom Anim* 2009;44 Suppl 2:141–7.
- [7] Davidson AP. Uterine and fetal monitoring in the bitch. *Vet Clin North Am Small Anim Pract* 2001;31:305-13.
- [8] Schröder M, Münnich A, Falkenberg U, Heuwieser W. Tocodynamometry as a non-invasive method for monitoring labour patterns, delivery and dystocia in the dog to reduce neonatal mortality. *Reprod Dom Anim* 2006;41 Suppl 1:31.
- [9] Groppetti D, Pecile A, Del Carro AP, Copley K, Minero M, Cremonesi F. Evaluation of newborn canine viability by means of umbilical vein lactate measurement, apgar score and uterine tocodynamometry. *Theriogenology* 2010;74:1187-96.
- [10] Ayres-de-Campos D, Spong CY, Chandrachan E, FIGO Intrapartum Fetal Monitoring Expert Consensus Panel. FIGO consensus guidelines on intrapartum fetal monitoring: Cardiotocography. *Int J Gynaecol Obstet* 2015;131:13-24.
- [11] Miles AM, Monga M, Richeson KS. Correlation of external and internal monitoring of uterine activity in a cohort of term patients. *Am J Perinatol* 2001;18:137-40.
- [12] van der Weyden GC, Taverne MA, Okkens AC, Fontijne P. The intra-uterine position of canine foetuses and their sequence of expulsion at birth. *J Small Anim Pract* 1981;22:503-10.
- [13] Euliano TY, Nguyen MT, Darmanjian S, McGorray SP, Euliano N, Onkala A, et al. Monitoring uterine activity during labor: a comparison of 3 methods. *Am J Obstet Gynecol* 2013;208:66.e1-6.
- [14] Macones GA, Hankins GD, Spong CY, Hauth J, Moore T. The 2008 National Institute of Child Health and Human Development workshop report on electronic fetal monitoring: update on definitions, interpretation, and research guidelines. *Obstet Gynecol*. 2008;112:661-6.
- [15] Nurani R, Chandrachan E, Lowe V, Ugwumadu A, Arulkumaran S. (2012). Misidentification of maternal heart rate as fetal on cardiotocography during the second stage of labour: the role of the fetal electrocardiograph. *Acta Obstet Gynecol Scand* 2012;91:1428-32.

- [16] Assali NS, Dasgupta K, Kolin A, Holms L. Measurement of uterine blood flow and uterine metabolism. V. Changes during spontaneous and induced labor in unanesthetized pregnant sheep and dogs. *Am J Physiol* 1958;195:614-20.
- [17] Sato M, Noguchi J, Mashima M, Tanaka H, Hata T. 3D power Doppler ultrasound assessment of placental perfusion during uterine contraction in labor. *Placenta* 2016;45:32-6.
- [18] Gibb DM, Arulkumaran S. Assessment and management of uterine contractions. *Baillière's Clin Obstet Gynaecol* 1987;1:111-29.
- [19] Leman H, Marque C, Gondry J. Use of the electrohysterogram signal for characterization of contractions during pregnancy. *IEEE Trans Biomed Eng* 1999;46:1222-9.
- [20] Sinnemaa L, Järvinen T, Lehmonen N, Mäkelä O, Reilas T, Sankari S, et al. Effect of insemination volume on uterine contractions and inflammatory response and on elimination of semen in the mare uterus. *J Vet Med A Physiol Pathol Clin Med* 2005;52:466–71.
- [21] Campbell ML, England GC. Effect of teasing, mechanical stimulation and the intrauterine infusion of saline on uterine contractions in mares. *Vet Rec* 2004;155:103-10.
- [22] Vlemminx MW, de Lau H, Oei SG. Tocogram characteristics of uterine rupture: a systematic review. *Arch Gynecol Obstet* 2017;295:17-26.



Figure 1. Positioning of the bitch and the probe for detecting uterine contractions using Sonicaid FM 820 CTG monitor.

Table 1. Observed uterine contractions during cardiotocography monitoring in different stages of parturition in 47 pregnant bitches.

NORM, normal parturition; CUI, complete uterine inertia; PUI, partial uterine inertia; OD, obstructive dystocia; MD, medically treated dystocia. Letters in superscript indicate a significant difference ($p < 0.05$) within the column.

Group		Individual monitoring time, min (mean)	Number of contractions (mean)	Mean number of contractions /10 min	Change of amplitude %-units (mean)	Interval between contractions, min (mean)	Duration of contractions, min (mean)
Prepartum (PRE, n=13)		8-170 (39)	0-3 (2.0)	0.7 ^a	10-15 ^a (13.8)	4-9 (6.5)	1-3 (2.1) ^a
First stage of parturition (FIRST, n=7)		7-165 (57)	0-11 (3.9)	0.9	10-30 (21.7)	4-21 (10.6)	1-4 (2.6)
Second stage of parturition (n=37)	NORM (n=17)	13-140 (86)	1-31 (8.3)	1.1 ^b	20-45 ^b (32.6)	1-14 (6.2)	2-5 (3.1) ^b
	CUI (n=3)	20-45 (32)	0	0	<10	-	<1
	PUI (n=8)	10-110 (42)	1-14 (5.0)	1.2 ^b	10-30 (17.5)	4-13 (7.3)	1-4 (2.3)
	OD (n=3)	20-45 (29)	3-4 (3.3)	1.3	20-40 ^b (30.0)	2-8 (5.0)	2-4 (3.0)
	MD (n=6)	14-56 (34)	1-7 (4.2)	1.3 ^b	15-45 ^b (29.1)	3-4 (3.5)	2-4.5 (3.0) ^b

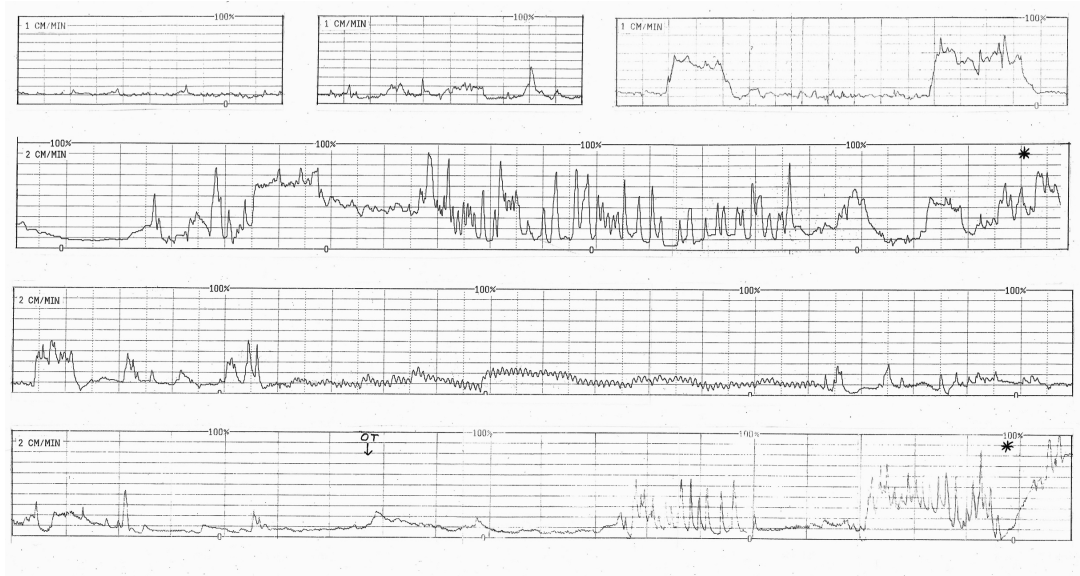


Fig. 2. Uterine activity presented as tocographs of relative pressure change (%-units): a) prepartum, no contractions; b) first stage of parturition, same bitch as in (a) but 2 days later, no clear contractions but change in uterine activity, first puppy was born 5 hours later; c) the second stage of normal parturition, peaceful dam, two contractions; d) second stage of normal parturition, restlessly panting dam, one clear contraction, strong abdominal straining, birth of the puppy is marked with an asterisk; e) uterine inertia, same bitch as in (d), 4 hours later, no response to oxytocin treatment given 5 min before onset of monitoring, Cesarean section eventually performed; f) uterine inertia, 12 min after oxytocin administration (marked with OT and arrow) last puppy born (marked with asterisk), abdominal straining and one clear contraction. Scale of the original graph is 1 cm/min in a-c and 2 cm/min in d-f.

Appendices



Synnyttävän koiran omistajalle

TUTKIMUS: Koiran polttoheikkous

Koiran dystokian eli synnytysvaikeuden taustalla voi olla useita tekijöitä kuten rotu, ruumiinrakenne, pentueen pieni tai suuri koko ja ensisynnyttäjän korkea ikä. Yksi yleisimmistä syistä koiran synnytysvaikeuteen on kohdun supistusten puuttuminen. Kohdun valmistautuminen synnytykseen on hormonien säätelemää ja synnytyksessä kohdun supistuksia lisää aivolisäkkeestä vapautuva oksitosiinihormoni. Tämän tutkimuksen tarkoituksena on selvittää mm. oksitosiinireseptorien toimintaa kohdussa erityisesti nartuilla, joilla ei ole ollenkaan supistuksia (primaari inertia uteri).

Tutkimukseen voivat osallistua kaikki yli 5 kg painavat nartut, joille joudutaan tekemään keisarileikkaus. Keisarileikkauksen yhteydessä koiraltanne otetaan kohdun seinämästä kudoksenäyte samasta viillosta, minkä kautta pennut nostetaan kohdusta. Myös istukasta otetaan näyte. Koiraltanne otetaan verinäyte, josta tutkitaan mm. oksitosiinihormonin pitoisuutta veressä. Koiranne kohdun supistuvuutta voidaan myös tutkia vatsan ympärille laitettavalla monitorointilaitteella. Tutkimukseen liittyvät näytteenotot ja toimenpiteet eivät haittaa emää tai pentuja. Tutkimuksessa ovat mukana Yliopistollinen eläinsairaala ja Mäntsälän eläinlääkäriasema.

Mikäli haluatte osallistua tutkimukseen, allekirjoittakaa oheinen suostumus ja täyttäkää kyselylomake. Lomakkeet voitte palauttaa henkilökunnalle. Tietoja voi myöhemmin täydentää sähköpostitse (tuire.tamminen@helsinki.fi). Tarvittaessa lisätietoja tutkimuksesta voi myös soittaa alla olevaan numeroon. Tiedot käsitellään luottamuksellisesti. Teidän tai koiranne yksilötietoja ei julkaista tutkimusraportissa.

Tutkimukseen liittyvät toimenpiteet ja näytteenotot eivät lisää kustannuksia. Käynti, toimenpiteet, lääkitykset ja mahdolliset päivystyslisät laskutetaan normaalisti.

Avustanne kiittäen

Tuire Tamminen, ELL
Eläinlääketieteellinen tiedekunta
Helsingin yliopisto
Leissantie 41
04920 SAARENTAUS
P. 050-4150196
Fax: 019-685 1181
tuire.tamminen@helsinki.fi

Outi Laitinen-Vapaavuori, prof.
outi.vapaavuori@helsinki.fi

Merja Dahlbom, ELT, dipl ECAR
Mäntsälän eläinlääkäriasema



HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

SUOSTUMUS OSALLISTUMISESTA KOIRAN POLTTOHEIKKOUS -TUTKIMUKSEEN

Minulle, _____ koiran omistajalle, on selitetty edellä mainitun tutkimuksen tarkoitus, tutkimusmenetelmät ja tutkimukseen liittyvät riskit.

Olen tietoinen siitä, että tutkimukseen osallistuminen on vapaaehtoista. Voin halutessani perua osallistumisen tutkimukseen ilman, että sillä on vaikutuksia koirani hoitoon tai asiakassuhteeseeni. Ymmärrän myös, että tiedot käsitellään luottamuksellisesti.

Suostun siihen, että koirastani poistettuja kudokset ja verinäytteitä voidaan käyttää tutkimustarkoituksiin ja siihen, että tulokset voidaan julkaista tieteellisessä kirjallisuudessa.

_____ / _____ 20____

Suostun osallistumaan tutkimukseen.

Omistajan allekirjoitus _____

Nimen selvennys _____



Tutkimus koiran polttoheikkoudesta

Tämän kyselyn tarkoituksena on selvittää Koiran polttoheikkous –tutkimukseen osallistuvien koirien taustatietoja. Suurimpaan osaan kysymyksistä vastataan rasti ruutuun - menetelmällä. Osaan kysymyksistä voi valita useamman vastausvaihtoehdon.

Vastatkaa kysymyksiin mahdollisimman tarkasti. Tarvittaessa käyttäkää paperin kääntöpuolta lisäselvitysten antamiseen. Lisätietoja voi myös myöhemmin lähettää sähköpostitse osoitteeseen: tuire.tamminen@helsinki.fi

Vastaukset käsitellään luottamuksellisesti. Tutkimusta julkaistaessa omistajan tai koiran yksilötietoja ei julkaista.

Lämmin kiitos vastauksestanne!

Omistajan nimi: _____
Osoite: _____
Puhelinnumero: _____
Sähköpostiosoite: _____

A. Perustiedot

- 1) Koiran rotu: _____ 2) Koiran kutsumanimi: _____
- 3) Koiran kennelnimi: _____
- 4) Koiran syntymäaika: _____ 5) Koiran paino ilman pentuja: _____ kg
- 6) Missä koira on syntynyt?
 - ☐ syntynyt Suomessa, ei ole käynyt ulkomailla
 - ☐ syntynyt Suomessa, käynyt ulkomailla, missä: _____
 - ☐ tuontikoira, syntynyt missä: _____

B. Aiemmat tiineydet

1) Kuinka monta aiempaa tiineyttä koirallanne on ollut?

- ☐ 0 (siirry kohtaan C)
☐ 1 tiineys, minkä ikäisenä: ____ vuotta
☐ 2 tiineyttä, minkä ikäisenä: ____ vuotta, ____ vuotta
☐ 3 tiineyttä, minkä ikäisenä: ____ vuotta, ____ vuotta, ____ vuotta
☐ 4 tiineyttä, minkä ikäisenä: ____ vuotta, ____ vuotta, ____ vuotta, ____ vuotta

2) Kuinka monta pentua kuhunkin pentueeseen on syntynyt?

- | 1. pentue | 2. pentue | 3. pentue | 4. pentue |
|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| <input type="checkbox"/> 1 | <input type="checkbox"/> 1 | <input type="checkbox"/> 1 | <input type="checkbox"/> 1 |
| <input type="checkbox"/> 2-4 | <input type="checkbox"/> 2-4 | <input type="checkbox"/> 2-4 | <input type="checkbox"/> 2-4 |
| <input type="checkbox"/> 5-9 | <input type="checkbox"/> 5-9 | <input type="checkbox"/> 5-9 | <input type="checkbox"/> 5-9 |
| <input type="checkbox"/> 10-16 | <input type="checkbox"/> 10-16 | <input type="checkbox"/> 10-16 | <input type="checkbox"/> 10-16 |

3) Ovatko aiemmat tiineysajat sujuneet normaalisti?

- ☐ kyllä
☐ ei

4) Mitä ongelmia aiempien tiineyksien aikana on ollut?

- ☐ tiineyden keskeytyminen
☐ emän sairastuminen, mihin: _____
☐ muu, mikä: _____

5) Ovatko aiemmat synnytykset sujuneet normaalisti?

- ☐ kyllä
☐ ei

6) Mitä ongelmia aiemmissa synnytyksissä on ollut?

- ☐ synnytys ei ole käynnistynyt
☐ hitaasti etenevä synnytys / heikot supistukset
☐ ahdas emä / suuri pentu
☐ epämuodostunut pentu
☐ pentu virheasennossa
☐ kohdussa kuollut / kuolleena syntynyt pentu
☐ muu, mikä: _____

7) Onko koiranne keisarileikattu?

- ☐ ei
☐ kerran
☐ kaksi kertaa
☐ kolme kertaa

C. Tämänhetkinen tiineys

- 1) Onko tähän tiineyteen johtanutta kiimaa kontrolloitu?

☐ ei
☐ kyllä, vaginan irtosolunäyte (ns. papa)
☐ kyllä, progesteroni (viimeisin ennen astutusta) pvm ja arvo: _____
☐ kyllä, irtosolunäyte ja progesteroni (viimeisin ennen astutusta) pvm ja arvo: _____

- 2) Kuinka monta kertaa koira on astutettu tähän kiimaan?

☐ kerran, pvm: _____
☐ kahdesti, pvm: _____
☐ kolme kertaa, pvm: _____
☐ neljä tai enemmän, pvm: _____

- 3) Kuinka monta kertaa koira on keinosiemennetty tähän kiimaan?

☐ kerran, pvm: _____
☐ kahdesti, pvm: _____
☐ kolme kertaa, pvm: _____
☐ neljä tai enemmän, pvm: _____

- 4) Arvio pentujen määrästä?

☐ ultraäänitutkimuksen avulla: _____ kpl
☐ röntgen-kuvauksen avulla: _____ kpl

- 5) Onko koiranne saanut tämän **tiineyden** aikana lääkityksiä? (Synnytykseen liittyvät lääkitykset kohdassa D 5)

☐ ei
☐ kyllä, mitä: _____

D. Synnytyksen käynnistyminen (tämänhetkinen tiineys)

- 1) Onko synnytyksen avautumisvaihe (läähättely, petaaminen ym.) alkanut?

☐ ei
☐ en osaa sanoa
☐ kyllä, päivämäärä: _____ Klo: _____

- 2) Kuinka kauan avautumisvaihe kesti ennen ensimmäisen pennun syntymää?

<input type="checkbox"/> en osaa sanoa	<input type="checkbox"/> 5-12 tuntia
<input type="checkbox"/> yhtään pentua ei ole syntynyt	<input type="checkbox"/> 12-24 tuntia
<input type="checkbox"/> alle 5 tuntia	<input type="checkbox"/> yli 24 tuntia: _____ tuntia

- 3) Syntyneet pennut:

Ensimmäisen pennun syntymäpäivä: _____ Klo: _____
Viimeisen pennun syntymäpäivä: _____ Klo: _____

- 4) Jos yhtään pentua ei ole syntynyt, onko selviä työntöjä nähty?

☐ ei
☐ kyllä, heikkoja, noin klo: _____ välisenä aikana
☐ kyllä, voimakkaita, noin klo: _____ välisenä aikana

- 5) Synnytyksen aikana annetut lääkkeet:

☐ kalkki, klo: _____ antotapa: _____
☐ oksitosiini, klo: _____ antotapa: _____
☐ muu, mikä: _____ klo: _____ antotapa: _____

E. Rotukohtaisia kysymyksiä

1) Esiintyykö mielestänne koiranne rodussa polttoheikkoutta?

- | | |
|--|--|
| <input type="checkbox"/> en osaa sanoa | <input type="checkbox"/> kohtalaisesti |
| <input type="checkbox"/> ei | <input type="checkbox"/> paljon |
| <input type="checkbox"/> vähän | |

2) Onko rotujärjestön tai vastaavan toimesta selvitetty rodun keisarileikkausten määrää?

- | | |
|--|--|
| <input type="checkbox"/> en osaa sanoa | |
| <input type="checkbox"/> kyllä, keisarileikkausten osuus prosentteina synnytyksistä: _____ | |
| <input type="checkbox"/> kyllä, mutta en tiedä prosenttiosuutta | |

Päiväys: _____._____. 20_____

Lomakkeen täyttäjän allekirjoitus:_____

Nimen selvennys:_____

